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The Lysosomal Targeting of AMPARs in Response to Amyloidopathy

The Lysosomal Targeting of AMPARs in Response to Amyloidopathy

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1 ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterised by cognitive impairment. Amyloid-β (Aβ), a peptide generated from the cleavage of amyloid precursor protein (APP), is a pathological hallmark of AD and believed to underlie the synaptic pathophysiology of the disease. Synaptic malformations are accompanied by electrophysiological phenotypes, including the inhibition of long term potentiation (LTP) and facilitation of long term depression (LTD). Furthermore, Aβ can disrupt α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) trafficking, a key determinant of synaptic strength and synaptic plasticity, by causing their removal from the synaptic surface. Based on the observation that GluA1 and GluA2 protein levels are reduced in AD post-mortem brains, this synaptic removal could be a result of degradation of these subunits.

A multitude of proteins interact with AMPARs to control their trafficking including cortactin, which maintains surface levels of GluA2-containing AMPARs by directing receptors away from lysosomes. This interaction is negatively regulated by Src family kinase (SFK) phosphorylation of cortactin. Since the SFK family demonstrates aberrant activity in response to Aβ, disruption of the cortactin-GluA2 interaction might be responsible for AMPAR trafficking abnormalities in the disease.

Here we show that the GluA2 but not GluA1 receptor subunit displays reduced expression in two models of amyloidopathy. Furthermore, overexpressing APP_{WT} in hippocampal neuronal cultures causes the targeting of GluA2-containing AMPARs to lysosomal compartments. An increase in the phosphorylation of the Y466 cortactin residue was also demonstrated in response to APP_{SWE} expression.

The results suggest that Aβ disrupts the trafficking of GluA2-containing AMPARs by directing receptors for lysosomal degradation. This could be a result of increased cortactin phosphorylation which disrupts the GluA2-cortactin interaction so that receptors cannot be directed away from lysosomes.

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A big thank you to the entire Hanley and Henley lab for such an entertaining and stimulating year. Thank you first and foremost to my supervisor Dr Jon Hanley for his invaluable help and guidance throughout the project, including his support and patience whilst I juggled thesis writing with full-time work! Thank you to Suko for running the whole lab so smoothly and meticulously and to Kev for his help and advice. An additional thank you to Siobhan for the wine, the sing-alongs and the friendship, she's made the whole experience a lot of fun!

Thank you to the girls of Bellevue for their support, especially when I was greeted by yet more darkness down the microscope with neurons that refused to be transfected, and to my family for their encouragement and for believing me when I insisted that I would eventually have a finished thesis.

Although I have decided that a PhD and career in research is not for me, I have thoroughly enjoyed my time in the lab and will take from it many valuable lessons as I start out my career as a medical writer.

Author’s declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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List of Abbreviations

Aβ: Amyloid-β

AD: Alzheimer's disease

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor

ANOVA: Analysis of variance

AP2: Adaptor protein 2

AP5: Amino-5-phosphonovaleric acid

APOE: Apolipoprotein E

APP: Amyloid precursor protein

APP_{MV}: MV-mutant APP

APP_{SWE}: Swedish-mutant APP

APP_{WT}: Wild-type APP

APS: Ammonium persulfate

Arp: Actin-related protein

β2AR: β2-adrenergic receptor

BACE1: β-secretase 1

BSA: Bovine serum albumin

CA1: Cornu ammonis area 1

CA3: Cornu ammonis area 3

CaM: calcium/calmodulin

CAMKII: calcium/calmodulin-dependent protein kinase II

cDNA: Complementary deoxyribonucleic acid

CI: Ca^{2+}-impermeable

CIE: Clathrin-independent endocytosis

CIP: Calf intestinal alkaline phosphatase

CME: Clathrin-mediated endocytosis

CP: Ca^{2+}-permeable

CRISPR: Clustered regularly interspaced short palindromic repeats

CTD: Carboxyl-terminal domain

ddH_2O: Deionised distilled water

DMEM: Dulbecco's Modified Eagle Medium

DNA: Deoxyribonucleic acid

dNTP: 2'-deoxynucleoside-5'-triphosphate

DPBS: Distilled phosphate buffered saline

EAAT1: Excitatory amino acid transporter 1

EAAT2: Excitatory amino acid transporter 2

ECL: Enhanced chemoluminescence

E.Coli: Escherichia coli

EDTA: Ethylenediamine tetra acetic acid

EE: Early endosome

EEA1: Early endosome antigen 1

EGFP: Enhanced green fluorescent protein

EH: Eps15-homology

eNMDAR: Extrasynaptic NMDAR

EPSC: Excitatory postsynaptic current

ER: Endoplasmic reticulum

Erk: Extracellular signal regulated kinase

ESCRT: Endosomal sorting complex required for transport

EZ: Endocytic zone

F-actin: Filamentous actin

FBS: Fetal bovine serum

G-actin: Globular actin

GAP43: Growth-associated protein 43

GEF: Guanine nucleotide exchange factor

GLR-1: Glutamate receptor 1

GPCR: G-protein coupled receptor

GRIP: Glutamate receptor interacting protein

GTP: Guanosine 5'-triphosphate

HBS: HEPES buffered saline

HBSS: Hank's balanced salt solution

HEK: Human embryonic kidney

HOPS: Homotypic fusion and vacuole protein sorting

HRP: Horseradish peroxidase

HS1: Hematopoietic cell-specific Lyn substrate 1

ICC: Immunocytochemistry

ILV: Intra-luminal vesicle

L2K: Lipofectamine 2000

LAMP1: Lysosomal-associated membrane protein 1

LB: Luria-Bertani

LBD: Ligand-binding domain

LE: Late endosome

LTD: Long-term depression

LTP: Long-term potentiation

MAGUK: Membrane-associated guanylate kinase

mGluR: Metabotropic glutamate receptor

mRNA: Messenger RNA

MVB: Multivesicular body

Nedd4-1: Neural precursor cell expressed, developmentally down-regulated 4-1

NEEP21: Neuronal early endosomal protein 21

NMDA: N-methyl-D-aspartate

NMDAR: N-methyl-D-aspartate receptor

NTA: N-terminal acidic

NTD: N-terminal domain

N-WASP: Neuronal Wiskott Aldrich syndrome protein

PACSIN: Protein kinase C and casein kinase II substrate in neurons

PAK: p21-activated kinase

PCR: Polymerase chain reaction

PDL: Poly-D-lysine

PEI: Polyethylenimide

PFA: Paraformaldehyde

PICK1: Protein interacting with C kinase 1

PIP(3)P: Phosphatidylinositol 3-phosphate

PKA: Protein kinase A

PKD: Protein kinase D

PP1: Protein phosphatase 1

Pr: Probability of transmitter release

PrPc: Cellular prion protein

PRR: Proline-rich region

PSD: Post-synaptic density

PSD-95: Post-synaptic density protein 95

PTMs: Post-translational modifications

PVDF: Polyvinylidene difluoride

Q: Glutamine

R: Arginine

Rab11-FIP2: Rab11-family interacting protein 2

RE: Recycling endosome

RNA: ribonucleic acid

SAP97: Synapse-associated protein 97

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM: Standard error of the mean

SFK: Src family kinase

SH3: Src homology 3

SNAP: Soluble NSF attachment protein

SNAP-23: Synaptosome-associated protein-23

SNARE: Soluble NSF attachment protein receptor

TAE: TRIS-acetate-EDTA

TARPs: Transmembrane AMPAR regulatory proteins

TBS-T: TRIS buffered saline with Tween

TEMED: Tetramethylethylenediamine

TMD: Transmembrane domain

TRIS: Tris(hydroxymethyl)-amino-methane

UPS: Ubiquitin proteasome system

UV: Ultra-violet

VCA: C-terminal verprolin-homology domain, cofilin-homology, and acidic region

VPS: Vacuolar protein sorting

WB: Western blotting

WT: Wild-type

Measurements

a.u. Arbitrary units

AU: Airy unit

bp: Base pairs

C: Celsius

DIV: Days *in vitro*

E: Embryonic day

g: Grams

kb: Kilobase

L: Litre

m: Metre

M: Molar

min: minutes

s: seconds

V: Volts

w/v: Weight per volume

xg: Times gravity

2 INTRODUCTION

2.1 SYNAPTIC PLASTICITY

2.1.1 Principles of synaptic plasticity

The primary method of communication between neurons within neuronal networks is via fast chemical synapses. An action potential originating in dendritic spines propagates down the axon to the pre-synaptic terminal where voltage-gated Ca^{2+} channels open in response to the depolarisation. Ca^{2+} ions entering the terminal trigger synaptic vesicles to secrete neurotransmitter which diffuses across the synaptic cleft and is recognised by receptors on the post-synaptic membrane thus initiating a response (Zucker and Regehr, 2002). The ability of this connectivity between a subset of neurons to be enhanced was first demonstrated over 70 years ago (Eccles, Katz and Kuffler, 1941) and since then much has been revealed about the giant feats of synaptic adaptability.

In 1953 Donald Hebb theorised that coincident firing between neurons strengthened their synaptic connections - according to his postulate 'cells that fire together, wire together' (Hebb, 1953). His pioneering work was the first predecessor of the concept we now refer to as synaptic plasticity: the modification of the strength of synaptic transmission in response to activity. It is now known that the neural activity induced by an experience has the ability to create new, eliminate old and modify existing connections between cells, and that this plays a central role in behavioural functions (Citri and Malenka, 2007). Synaptic plasticity has demonstrated functional roles in addiction (Mansvelder and McGehee, 2000; Faleiro, Jones and Kauer, 2004), fear conditioning (McKernan and Shinnick-Gallagher, 1997; Rumpel *et al*., 2005) and sensory cortex development (Allen, Celikel & Feldman, 2003; Frenkel and Bear, 2004). Perhaps most importantly, synaptic plasticity has been widely evidenced to be the cellular correlate of learning and memory (Morris *et al*., 1986; Nabavi *et al.,* 2014). Indeed, since the term engram was first coined in the early twentieth century as 'the enduring though primary latent modifications in the irritable substance produced by a stimulus' (Semon, 1921) it has become the principal belief that these 'modifications' underlying memory formation are stored across 'engram cells' or neuronal ensembles that are activated by learning, have enduring cellular changes as a consequence of learning, and whose reactivation by a part of the original stimuli delivered during learning results in memory recall (Tonegawa *et al*., 2015) and it is thought that synaptic plasticity may play an early role in establishing memory storage across these

ensembles. Considering synaptic plasticity serves such a wide variety of functions, it can be further categorised according to its temporal domain.

2.1.2 Long-term synaptic plasticity

The long-term plasticity of excitatory synaptic transmission can be explained by two phenomena commonly termed long-term potentiation (LTP) and long-term depression (LTD). These phenomena are responsible for the long-lasting rewiring of neural circuitry following prolonged coincident firing.

LTP, described as a persistent increase in synaptic strength, was first demonstrated experimentally in the early 1970s in the hippocampus when repetitive stimulation of the perforant path caused a potentiation of synaptic strength in granule cells of the dentate gyrus which lasted between the order of hours to days (Bliss and Gardner-Medwin, 1973; Bliss and Lømo, 1973). LTD, a persistent decrease in synaptic strength, was also first demonstrated in the hippocampus in cornu ammonis area 1 (CA1) cells following low frequency stimulation of the Schaffer collateral pathway (Dudek and Bear, 1992).

The hippocampus, in particular the Schaffer collateral pathway that runs from cornu ammonis area 3 (CA3) to CA1, remains the most extensively studied model for synaptic plasticity and is where the bulk of our knowledge on these phenomena has been derived. These insights can be applied to long-term plasticity at excitatory synapses throughout the mammalian brain, although there are several forms of LTP and LTD that have been established (Citri and Malenka, 2007). Indeed, plasticity can be induced by N-methyl-D-aspartate receptors (NMDARs) (Dudek and Bear, 1992; Malenka and Nicoll, 1993) group I metabotropic glutamate receptors (mGluRs) (Moult *et al* 2006; Moult *et al* 2008) and endocannabinoids (Gerdeman, Ronesi and Lovinger, 2002; Sjöström, Turrigiano and Nelson, 2003). Furthermore, the locus of expression of both LTP and LTD has been a topic of debate. Although the emerging consensus is that postsynaptic mechanisms predominate, evidence that mGluR LTD can result in an increase in both the paired-pulse ratio and the coefficient of variation of excitatory postsynaptic currents (EPSCs) (Fitzjohn *et al.,* 2001) and that LTP induction can result in changes in paired-pulse facilitation (Lauri *et al.*, 2007) glutamate release (Dolphin, Errington and Bliss, 1982) and vesicular fusion (Malgaroli *et al.*, 1995) are indicative of pre-synaptic processes. It has been suggested that a developmental shift in plasticity expression mechanisms could account for the controversy (Nosyreva and Huber, 2005) as well as the involvement of a retrograde messenger linking pre-synaptic expression with post-synaptic induction (Feinmark *et al*., 2003). Furthermore,

differences in the stimulation responsible for triggering LTP can also influence locus of LTP expression, with a meta-analysis demonstrating that LTP is significantly more likely to have a presynaptic component of expression when induced by tetanic stimulation rather than by pairing (Padamsey and Emptage, 2014).

NMDAR-dependent LTP and LTD in the CA1 region of the hippocampus are the most extensively studied forms of plasticity and there is much evidence suggesting their involvement in long-term memory. Thus this thesis will focus on these processes of long-term plasticity.

2.1.3 NMDA receptor-dependent LTP and LTD

The ionotropic glutamatergic receptors NMDARs and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs) are often co-localised on dendritic spines at excitatory synapses. AMPARs are responsible for the majority of post-synaptic depolarisation during basal synaptic stimulation, with permeability to both Na^+ and K^+ ions. In contrast the NMDAR exhibits a Mg^{2+} block at negative membrane potentials (Mayer, Westbrook and Guthrie, 1984; Nowak *et al*; 1984) and therefore requires both glutamate binding and post-synaptic depolarisation in order to conduct ions into the cell – it is a 'coincidence detector'. Subsequently, NMDARs only contribute to the post-synaptic response once the cell is depolarised. In addition to Na^+ and K^+, NMDARs are permeable to Ca^{2+} and it is this post-synaptic rise in Ca^{2+} concentration that is critical for the induction of both LTP and LTD.

The magnitude of the post-synaptic Ca^{2+} signal determines the direction of plasticity, with LTD requiring a modest increase in Ca^{2+} concentration and LTP requiring an increase above a critical threshold (Cho *et al.,* 2001) (figure 2-1). Moreover, there is evidence that the temporal properties of the Ca^{2+} current may also be important, since the direction of synaptic change can be reversed depending on the order of the pre- and post-synaptic spike (Bi and Poo, 1998).

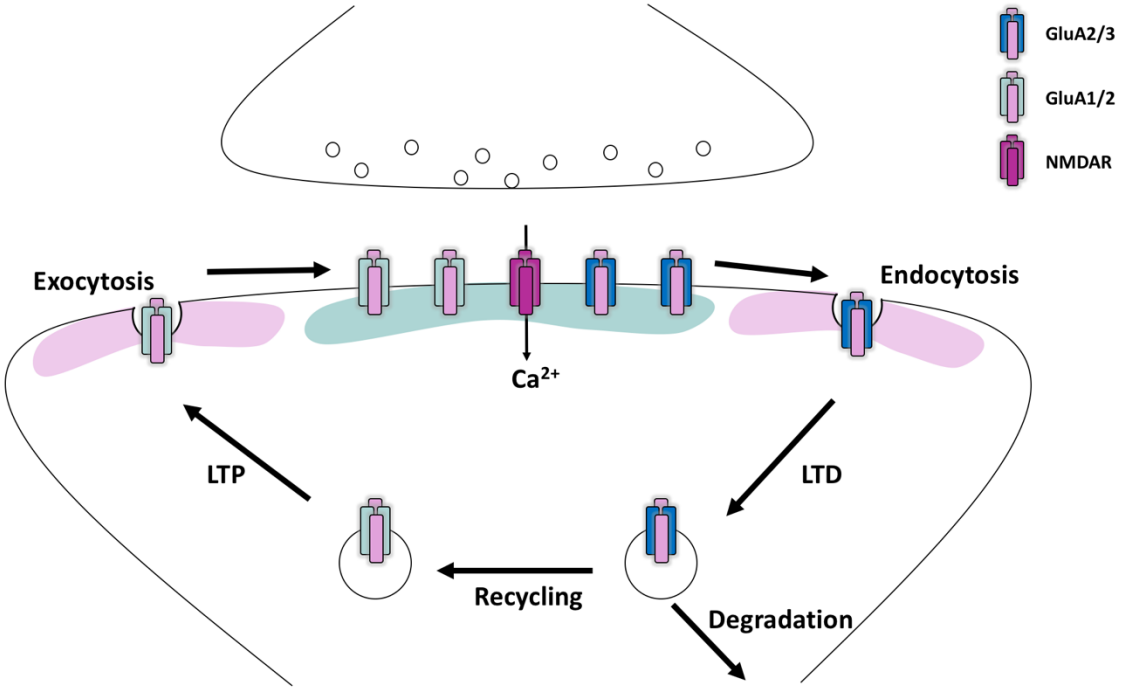

Figure 2-1 NMDAR-dependent bi-directional plasticity evoked by Ca^{2+} transients.

Post-synaptic depolarisation coupled with pre-synaptic neurotransmitter release results in NMDAR channel opening and the influx of Ca^{2+} ions. The strength and duration of the Ca^{2+} signal underlies activation of a series of signalling cascades and determines which form of plasticity prevails. A low Ca^{2+} signal culminates in LTD and results in a series of pre- and post-synaptic modifications including the internalisation of AMPARs from the post-synaptic membrane. Strong Ca^{2+} signals however, culminate in LTP and the insertion of AMPARs at the post-synapse.

In order to trigger an increase in Ca^{2+} above a critical threshold for LTP, a number of experimental induction protocols exist including high-frequency and theta-burst stimulation. With the use of these protocols, it has been widely evidenced that calcium/calmodulin (CaM)-dependent protein kinase II (CaMKII) is primarily responsible for transducing the Ca^{2+} signal into a long-lasting increase in synaptic strength. Indeed, CaMKII undergoes autophosphorylation following LTP induction (Fukunaga, Muller and Miyamoto 1995) and LTP induction was prevented both in knock-in mice in which the CaMKII autophosphorylation site was lacking and in CA1 pyramidal cells following intracellular injection of the CaMKII inhibitor H-7 (Malenka *et al* 1989)**.** Following CaMKII activation, the major expression mechanisms of LTP are postsynaptic, involving changes in the biophysical properties of AMPARs, as well as their trafficking. Indeed, LTP induction increases the single channel conductance of AMPARs, and CaMKII can phosphorylate Ser-831 on the C terminus of GluA1 to increase single channel conductance of homomeric GluA1 receptors (Derkach, Barria and Soderling, 1999; Lee *et al*; 2000). In terms of trafficking, CaMKII is required to drive AMPARs into synapses

(Hayashi *et al*., 2000), possibly via phosphorylation of transmembrane AMPAR regulatory proteins (TARPs), which aid the binding of AMPARs to 'slot proteins', thus trapping them inside the post-synaptic density (Tsui and Malenka, 2006; Tomita et al 2005).

The typical protocol for triggering a modest rise in Ca^{2+} concentration and thus LTD is with the use of low-frequency stimulation. It has been demonstrated that this signal is translated through the activation of a protein phosphatase cascade, consisting of the calcium/calmodulin dependent phosphatase calcineurin and protein phosphatase 1 (PP1). Consistent with this, postsynaptic inhibition of both of these phosphatases prevents the induction of LTD (Kirkwood and Bear, 1994; Morishita *et al*., 2001) and loading PP1 into CA1 pyramidal cells enhances LTD (Morishita *et al*., 2001). These phosphatases are thought to once again alter the trafficking and biophysical properties of AMPARs. Studies have indicated dephosphorylation of Ser-845 in response to LTD induction (Lee *et al*., 1998, 2000) which could be responsible for a decrease in AMPAR open channel probability (Banke *et al*., 2000). Furthermore, AMPARs undergo clathrin-mediated endocytosis (CME) following LTD induction (Ashby *et* al., 2004) which is regulated by Ca^{2+}-dependent dephosphorylation **(**Beattie *et al.,* 2000). Consistent with the contribution of 'slot proteins' in regulating AMPAR surface levels, it has been reported that LTD-induced endocytosis could be regulated by PP1-dependent dephosphorylation of stargazin, a TARP, allowing AMPARs to diffuse out of the post-synaptic density (PSD) (Tomita *et al*., 2005).

Given that LTP was first demonstrated in the hippocampus, and that this structure has been established to be critical for declarative memory (Squire, Stark and Clark, 2004), there have been major efforts in determining a role for hippocampal LTP in memory formation.

2.1.4 Plasticity and learning

Lesion studies have demonstrated impaired spatial navigation in rodents in a water maze task following hippocampal lesions (Morris *et al*., 1982) and rodents with subfield lesions to both CA1 and CA3 showed difficulty with episodic spatial memory processing (Hunsaker, Lee and Kesner, 2008). Pharmacological blockade and gene knockout studies suggest a necessity for LTP in hippocampal spatial learning since the NMDAR antagonist amino-5-phosphonovaleric acid (AP5) (Morris *et al.,* 1986) and genetic ablation of the NMDAR in CA1 and CA3 pyramidal cells (Tsien Huerta and Tonegawa, 1996; Nakazawa *et* al., 2002) inhibit both LTP induction and spatial learning. Perhaps the most convincing evidence for a role of synaptic plasticity in hippocampal-dependent learning are studies demonstrating that learning induces LTP. Indeed, during an inhibitory avoidance task, LTP was recorded in CA1 pyramidal cells *in vivo* demonstrating the patterns of activity

generated during learning to be consistent with LTP (Whitlock *et* al., 2006). As will be discussed, since plasticity has such major roles in learning and memory, detriment to these phenomena in disorders such as Alzheimer's disease (AD) have severe implications on cognitive processes.

As a major target of the kinases and phosphatases involved in plasticity, AMPARs and their involvement and regulation in both basal and activity-dependent neuronal function will be described below.

2.2 AMPARs

2.2.1 Glutamate receptors

There are two broad classifications of glutamate receptor families: ionotropic receptors, which exert their effects by conducting ions through a central channel pore; and metabotropic receptors which are coupled to second-messenger systems via heterotrimeric guanosine 5'-triphosphate (GTP)-binding proteins. The family of ionotropic glutamate receptors is classified into three major pharmacological sub-families defined by their most selective agonists: AMPA, NMDA and kainate (Molnar, 2018), and another subtype, the δ glutamate receptor family, whose physiological functions and endogenous ligands have only recently come to light (Yuzaki and Aricescu, 2017). The mGluRs comprise eight different subtypes, which are classified into three groups based on sequence homology, intracellular signal transduction and pharmacological properties (Niswender and Conn, 2010).

All ionotropic glutamate receptors share common membrane topology, but the overwhelming majority of fast excitatory transmission in the central nervous system is mediated by AMPARs. Changes to the composition and properties of these receptors at the post-synaptic membrane significantly modify synaptic function and neuronal connectivity.

2.2.2 AMPAR structure, composition and assembly

AMPARs are tetrameric assemblies of four subunits, each of which consists of four discrete semiautonomous domains: the extracellular N-terminal domain (NTD), the extracelluar ligand-binding domain (LBD), the transmembrane domain (TMD), and the intracellular carboxyl-terminal

domain (CTD) (Traynelis *et al*., 2010) (figure 2-2).

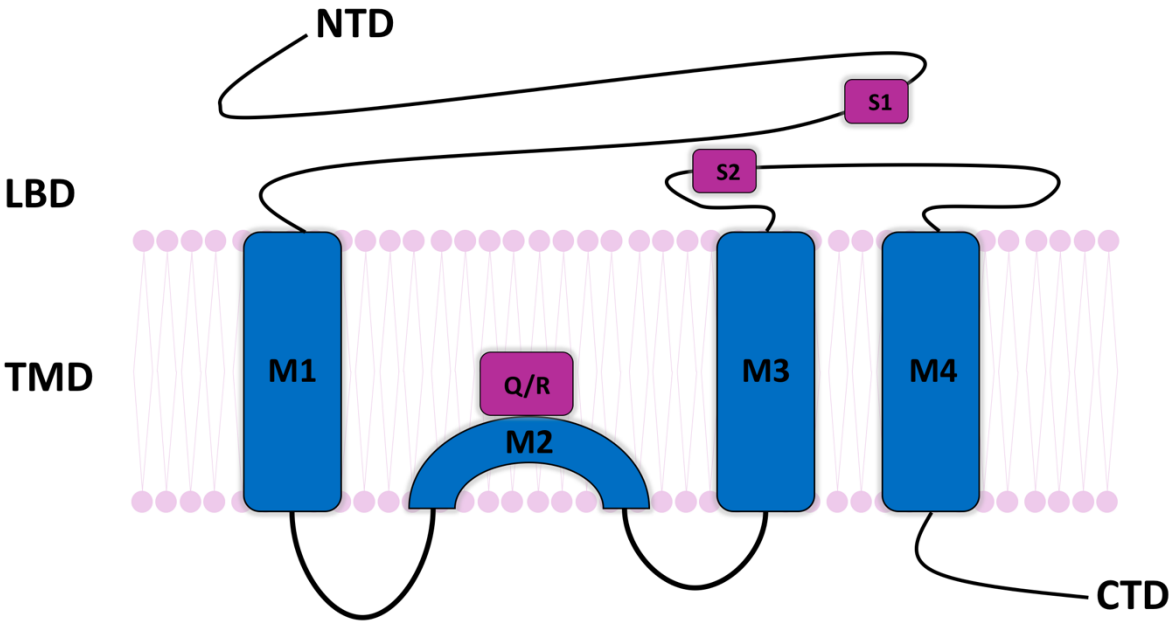

Figure 2-2 Topological organisation of an AMPAR subunit.

S1 and S2 are within the extracellular LBD, M1, 3, 4 and the re-entrant loop M2 are all part of the TMD. At the apex of the loop resides the GluA2 Q/R alternative splice region.

The AMPAR subunits, GluA1-GluA4, are preferentially assembled as heteromers in various combinations but can also form functional homomers. In mature hippocampal pyramidal neurons, the majority of AMPARs assemble as GluA1/2 or GluA2/3 heterodimers (Wenthold *et al*., 1996). The intracellular CTD of AMPAR subunits can be divided into short or long tails. GluA1 and GluA4 are long-tailed subunits, however GluA4 is primarily expressed early in development (Zhu *et al*., 2000) and is absent from most excitatory pyramidal neurons. GluA2 and GluA3 are short-tailed subunits. It has been suggested that long-tailed subunits are targeted to synapses in response to neuronal activity whilst short-tailed subunits are targeted constitutively to maintain AMPAR numbers (Hayashi *et al*., 2000; Shi *et al*., 2001). However, these conclusions could be oversimplified since many of these earlier studies rely on the overexpression of GluA1 or GluA2 in hippocampal slices, resulting in the biased synthesis of homomeric receptors.

AMPAR assembly occurs in the endoplasmic reticulum (ER) and, in common with K^+ channels, the receptors assemble as a dimer of dimers. Dimer affinities of AMPAR subunits range from low nanomolar to low micromolar and the formation of the initial dimer is driven by the NTD (Rossman *et al*., 2011; Zhao *et al*., 2016). Subunits possess a high affinity for NTD self-assembly which results in

the initial formation of homodimers (Herguedas et al 2013) before a reshuffling into heterodimers occurs based on affinities for different subtypes. The NTD of GluA1 for example, has an affinity for GluA2 that is more than 200-fold greater than any other subunit partners, favouring GluA1/2 heterodimers. Furthermore, Q/R editing impedes GluA2 homotetramerisation and facilitates ER retention (Greger, Khatri and Ziff, 2002) increasing its availability for subunit incorporation. Consistent with this, in CA1 pyramidal neurons, receptors are almost exclusively GluA2 containing heterotetramers and therefore impermeable to Ca^{2+} (Lu et al., 2009). Following dimerisation, subsequent tetramerisation occurs through interactions between the LBD and TMD (Ayalon and Stern-Bach, 2001).

2.2.3 AMPAR trafficking

The trafficking of AMPARs is a highly dynamic process and is regulated by post-translational modifications (PTMs) in the CTD and by interacting proteins specific for each subunit. As described in section 2.1.4, changes in the number and properties of AMPARs is a major mechanism underlying long-term plasticity of synapses, with increases in AMPAR number and function resulting in the LTP of synaptic strength, and removal of AMPARs in LTD (Shepherd and Huganir, 2007).

2.2.3.1 Subunit dependent trafficking

Previously described in section 2.2.3, there are two distinct populations of AMPARs in the adult hippocampus, GluA1/2 and GluA2/3, with long-tailed subunits targeted to synapses in response to neuronal activity whilst short-tailed subunits are targeted constitutively (Greger *et al*., 2003). The trafficking properties of long-tailed subunits predominate over short-tailed, thus GluA1/2 heteromers exhibit the trafficking properties of the GluA1 subunit (Greger *et al*., 2003). Specifically, of the long- and short-tailed subunits, GluA1 and GluA2 have been demonstrated to govern AMPAR trafficking rules. Consistent with this, the GluA2 subunit is constitutively inserted into synapses under basal conditions and largely determines activity-dependent AMPAR endocytosis (Shi *et al*., 2001). In contrast, the GluA1 subunit undergoes activity-dependent exocytosis into the post-synaptic membrane following LTP protocols and is constitutively internalised (Hayashi *et al*., 2000; Passafaro, Piëch and Sheng, 2001). This gives rise to a model in which GluA1/2 AMPARs are delivered to

synapses in response to LTP, constitutively replaced by GluA2/3 receptors to maintain the net increase in AMPAR numbers, and these GluA2/3 subunits are internalised following LTD activity.

It must be noted that there has been scrutiny of this model. Despite the prominent hypothesis that AMPARs composed of both GluA1 and GluA2 are incorporated into the synapse following LTP, later work proposed a slight variation to this mechanism. Indeed, it was demonstrated that an increase in the rectification of AMPAR-mediated EPSCs was observed for up to 25 minutes following LTP induction, indicating the transient incorporation of Ca^{2+} permeable (CP)-AMPARs into the post-synaptic membrane (Plant *et al*., 2006). It is believed these GluA2-lacking CP-AMPARs define an initial status of LTP which is labile and reversible, likely corresponding to an experience that is never laid down as a memory (Yang, Wang and Zhou, 2010). A further increase in Ca^{2+} concentration then drives the replacement of these receptors with Ca^{2+} impermeable (CI)-AMPARs to stabilise LTP and confer longer lasting plastic changes. However, the transient recruitment of CP-AMPARs during LTP is highly contentious and has not been reproduced by some labs (Adesnik and Nicoll, 2007; Gray *et al*., 2007). Further investigation is needed to determine whether both forms of LTP coexist and under what conditions they are selectively induced.

2.2.3.2 Exocytosis and synaptic stabilisation

The delivery of AMPARs to the synapse is believed to occur via soluble N-ethylmaleimide sensitive factor (NSF) attachment protein (SNAP) receptor (SNARE)-mediated exocytosis. Both myosin Va and Vb have been identified as the motor proteins responsible for AMPAR delivery. Myosin Vb associates with endosomes via the Rab11/Rab11-family interacting protein 2 (Rab11-FIP2) adaptor complex following an increase in Ca^{2+} concentration (Wang *et al*., 2008) whereas myosin Va associates with AMPARs directly (Correia *et al*., 2008). It has been elucidated that synaptosome-associated protein (SNAP)-23, consistent with being highly enriched in dendritic spines, could be the v-SNARE on the vesicular membrane responsible for glutamate receptor insertion events (Suh *et al*., 2010) and syntaxin-4 the t-SNARE directing membrane fusion to exocytic domains where it is enriched (Kennedy *et al*., 2010). However, contrary to this, work from another lab suggests syntaxin-3 and SNAP-47 are responsible for regulated AMPAR exocytosis during LTP but are not absolutely required for constitutive basal AMPAR exocytosis (Jurado *et al*., 2013).

AMPARs are inserted into the plasma membrane at extra-synaptic sites lateral to the post-synaptic membrane, and then lateral diffusion is required both for activity dependent AMPAR delivery

(Borgdorff and Choquet, 2002) and for the constitutive trafficking of AMPARs (Ashby *et al*., 2006) to dendritic spines. There is also evidence that, following LTP stimulation, recruitment of AMPARs by lateral movement is needed for the earlier phase of synaptic potentiation, and then LTP maintenance is sustained primarily by an intracellular reserve pool that replenish the extra-synaptic sites (Penn *et al*., 2017).

There exist a multitude of proteins whose job it is to stabilise AMPARs following exocytosis into the post-synaptic membrane. NSF is an ATPase that binds to the CTD juxtamembrane region of the GluA2 subunit to mediate constitutive AMPAR trafficking and stabilisation at synapses. Indeed, blocking the NSF-GluA2 interaction with the use of decapeptides results in a rundown of basal AMPAR synaptic currents (Nishimune *et al*., 1998). This is because NSF prevents the internalisation of GluA2 heteromers by blocking the interaction of GluA2 with protein interacting with C kinase 1 (PICK1) (Hanley *et al*., 2002) and antagonising the GluA2 interaction with the endocytic heterotetrameric adaptor protein 2 (AP2) (Lee *et al*., 2002). Protein 4.1N is a cytoskeletal scaffold protein that binds to the membrane proximal region of the GluA1 CTD to regulate activity-dependent GluA1 surface expression. Knockdown of protein 4.1N decreases the frequency of insertion of super-ecliptic pHluorin tagged GluA1 subunits and impairs LTP maintenance (Lin *et al*., 2009). Additionally, synapse-associated protein 97 (SAP97) is a member of the membrane-associated guanylate kinase (MAGUK) protein family, that also binds to the GluA1 subunit and increases AMPAR function at the post-synaptic membrane (Rumbaugh *et al*., 2003) and is thought to regulate activity-dependent trafficking during LTP (Mauceri *et al*., 2004) although this evidence remains controversial (Howard *et al*., 2010).

Perhaps among the most important partners to regulate AMPAR stability at the post-synaptic membrane are a family of transmembrane proteins called TARPs that are intrinsic partners of the AMPAR complex and thus termed auxiliary subunits (Opazo, Sainlos and Choquet, 2012). As mentioned in section 2.1.4, one member of the TARP family, stargazin, has been demonstrated to aid the binding of AMPARs to 'slot proteins' and trap them inside the PSD. The slot hypothesis states that the major determinant for synaptic strength is the number of available 'slots' available to anchor AMPARs on the post-synaptic membrane (Malinow, 2003). Post-synaptic density protein 95 (PSD-95), a MAGUK scaffolding protein, has been suggested as the most likely candidate to define a 'slot', by trapping the stargazin/AMPAR complex through an interaction with the stargazin PDZ binding motif. Consistent with this, studies with a stargazin mutant lacking the PDZ binding motif have demonstrated the run-down of basal excitatory synaptic transmission, suggesting lateral diffusion of AMPARs away from the synapse (Schnell *et al*., 2002; Bats, Groc & Choquet, 2007). Evidence demonstrates that AMPARs diffuse in the neuronal membrane together with stargazin

(Bats, Groc & Choquet, 2007), thus it is suggested that LTP occurs when more PSD-95 slots become available for stargazin/AMPAR complexes (Schnell *et al*., 2002). This could be via phosphorylation of the Ras/Rap GTPase-activating protein (GAP) SynGAP, which restricts binding to the PDZ domains of PSD-95 unless phosphorylated by CaMKII or Polo-like kinase-2 (PLK2), whereby its affinity for the PDZ domains decreases which frees up PSD-95 for occupancy by other proteins (stargazin/AMPAR) (Walkup *et al*., 2016). Alternatively, LTP could occur via the recruitment of additional stargazin-AMPAR complexes (Tomita et al 2005) to fill existing PSD-95 slots (Shen and Hoogenraad, 2007).

2.2.3.3 Endocytosis, sorting and recycling

The primary mechanism for the activity-dependent removal of AMPARs during LTD is believed to be CME. There are several lines of support for this, including evidence that both AMPAR-endocytosis and LTD are blocked by inhibitors of the dynamin-amphiphysin (Man et al 2000) and the AP2-GluA2 interaction (Lee et al 2002). In contrast, it has been suggested that constitutive internalisation may occur via clathrin-independent endocytosis (CIE). Indeed, constitutive AMPAR trafficking is regulated by a pool of filamentous actin (F-actin) but does not require dynamin or clathrin function (Glebov *et al*., 2015; Fujii, Tanaka and Hirano, 2017). Endocytosis of AMPARs is thought to occur at endocytic zones (EZs) adjacent to the PSD which are held in position via an interaction between dynamin-3 and the post-synaptic scaffold complex Homer/Shank (Lu *et al*., 2007).

In order for AMPARs to be internalised they must first dissociate from post-synaptic scaffolds. Glutamate receptor interacting protein (GRIP) can bind directly to GluA2/3 CTDs through their fourth and fifth PDZ domains to maintain AMPAR surface expression (Dong *et al*., 1997; Srivastava *et al*., 1998). The binding of GluA2 to GRIP is disrupted when GluA2 is phosphorylated on Ser-880 and Tyr-876 (Chung *et al*., 2000; Hayashi and Huganir, 2004). Crucially, hippocampal LTD is reliant on Ser-880 phosphorylation, thus detachment of GluA2 from GRIP has been hypothesised to be an expression mechanism of LTD resulting in the internalisation of AMPARs (Seidenman *et al*., 2003). Consistent with this, the dissociation of GRIP from GluA2 allows PICK1 to bind, which is an essential facilitator in the endocytosis (Fiuza *et al*., 2017) and restriction of recycling (Lin and Huganir, 2007; Citri *et al*., 2010) of GluA2-containing AMPARs. Similarly, the NSF-GluA2 interaction must be interrupted during LTD to favour PICK1-GluA2 interactions and AMPAR endocytosis (Hanley *et al*., 2002). The PICK1-GluA2 interaction is sensitive to Ca^{2+} in a biphasic manner, peaking at 15µM. This function of PICK1 is essential for regulating AMPAR trafficking since it has been demonstrated that overexpression of a PICK1 mutant insensitive to Ca^{2+} occludes NMDAR-dependent AMPAR endocytosis thus rendering

GluA2 internalisation insensitive to the presence of a Ca^{2+} signal (Hanley and Henley 2005). Furthermore, the presence of a BAR domain makes PICK1 a likely candidate to define an endocytic accessory protein that can transduce NMDAR stimulation and Ca^{2+} entry into the modulation of endocytic machinery. Consistent with this, PICK1 successfully drives AMPAR endocytosis by associating with clathrin-coated pits, interacting with AP2 to target GluA2 containing AMPARs to EZs and promoting dynamin polymerisation, all of which are increased following NMDAR stimulation (Fiuza *et al*., 2017).

Once internalised, AMPARs are trafficked to early endosomes (EEs). From here they can take one of three distinct trafficking pathways: recycling back to the post-synaptic membrane via recycling endosomes (REs) (van der Sluijs and Hoogenraad, 2011); maturation into late endosomes (LEs) and subsequently fusion with lysosomes for degradation (Hu *et al*., 2015); or retrograde trafficking back to the *trans*-Golgi network for further PTMs (Burd and Cullen, 2014) (figure 2-3).

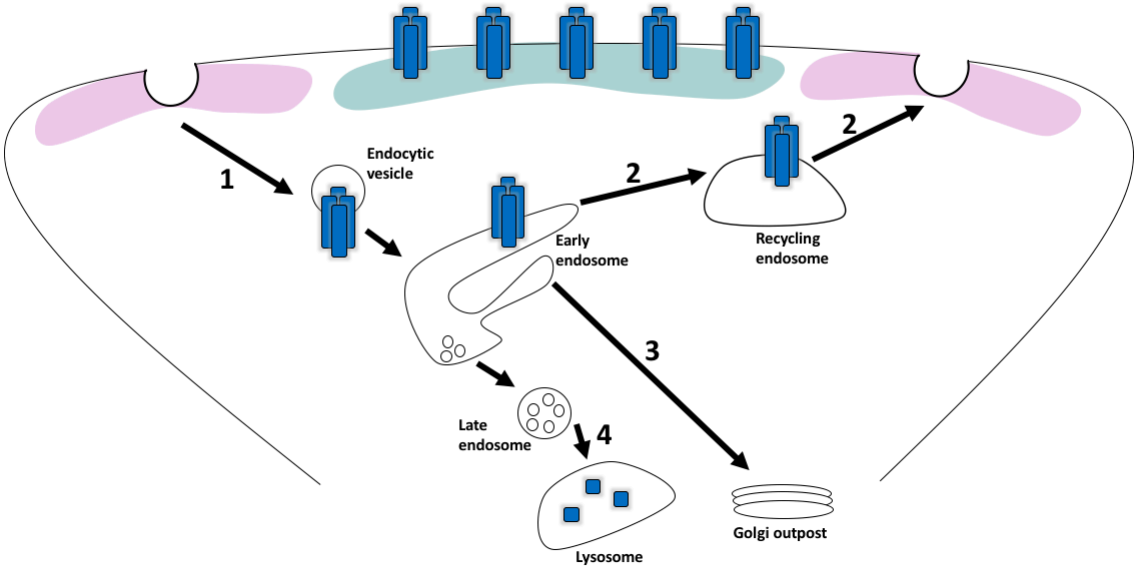

Figure 2-3 AMPAR trafficking pathways.

AMPARs (blue rectangles) are inserted into the plasma membrane at extra-synaptic sites (pink shaded area) before lateral diffusion to the PSD (blue shaded area) where they are caught and stabilised. AMPARs can then undergo endocytosis (1) and trafficking to EEs. From here they are sorted into several trafficking pathways. Receptors can recycle back to the plasma membrane via REs where they undergo exocytosis at extra-synaptic sites (2). AMPARs can also be retrogradely trafficked back to the trans-golgi network (3). Alternatively, vesicular domains on EEs can mature into LEs and fuse with lysosomes resulting in receptor degradation.

EEs function by sorting cargo into distinct endosomal membrane domains that undergo budding and fission and give rise to an array of tubulo-vesicular structures that transport cargo (Grant and

Donaldson, 2009). These micro-domains are characterised by varying morphologies and molecular compositions (Hirling, 2009). AMPAR entry into EEs requires Rab5 and EE antigen 1 (EEA1), a vesicle tethering protein that associates with phosphatidylinositol 3-phosphate (PIP(3)P) in the EE membrane (Gaullier *et al*., 2000) and binds Rab5 on an endocytic vesicle to facilitate the incorporation of cargo (Simonsen *et al*., 1998). Neuronal early endosomal protein 21 (NEEP21) also localises to EEs where it associates with GRIP (Steiner *et al*., 2005). Indeed, in addition to the plasma membrane, GRIP is also detected on internal compartments where it can be distinguished by its differential palmitoylation (DeSouza *et al*., 2002). It has been suggested that the NEEP21-GRIP interaction has a direct involvement in the correct sorting of GluA2-containing AMPARs back to the plasma membrane (Steiner *et al*., 2005).

Tubular domains on EEs are believed to give rise to REs, which have a tubular morphology and are positive for Rab11 (Ullrich *et al*., 1996) and the SNARE protein syntaxin 13 (Prekeris et al 1998). RME-1, a member of the Eps15-homology (EH)-domain proteins, has also been shown to play a direct role in endocytic recycling (Grant *et al*., 2001). Additionally, PICK1, as well as its role in endocytosis, has demonstrated involvement in the endosomal sorting of GluA2-containing AMPARs. Indeed, it was shown that: interference of the PICK1-ABP/GRIP interaction impaired GluA2 recycling and thus reduced surface levels of GluA2 (Lu and Ziff, 2005); neurons lacking PICK1 demonstrated accelerated GluA2 recycling after NMDAR activation (Lin and Huganir, 2007); PICK1 binding to the protein kinase C and casein kinase II substrate in neurons (PACSIN) regulated the rate of GluA2 recycling (Wigado *et al*., 2016); and that PICK1 was an essential component of the mechanism that underlies lysosomal targeting of GluA2-containing AMPARs caused by oxygen/glucose deprivation (Koszegi, Fiuza and Hanley, 2017).

2.2.3.4 Degradation

Cargo marked for degradation are sequestered into vesicular structures that invaginate from the endosomal membrane to form intra-luminal vesicles (ILVs), catalysed by the endosomal sorting complex required for transport (ESCRT) machinery (Huotari and Helenius, 2011). Once sorted into these bulbous regions of the EE, they rapidly acidify and mature into LEs (Babst, 2011) or multivesicular bodies (MVBs) if they have multiple ILVs (Hurley, 2008). Maturation of EEs into LEs also results from the re-modelling of membrane identity by the co-ordinated exchange of Rab5 for Rab7. This conversion requires the class C vacuolar protein sorting/homotypic fusion and vacuole protein sorting (VPS/HOPS) complex, a Rab7 guanine nucleotide exchange factor (GEF) (Rink *et al*.,

2005). LEs then fuse with the lysosome leading to the formation of an endolysosome, within which the cargo-enriched ILVs are degraded by a series of hydrolyses and lipases (Saftig and Klumperman, 2009).

As well as lysosomal degradation, AMPARs can be degraded in the proteasome (Zhang *et al.,* 2009*)*. Both fates are regulated by the ubiquitin system. Indeed, ubiquitination of glutamate receptors was first demonstrated in the C. *elegans* receptor glutamate receptor 1 (GLR-1), which showed increased abundance at the synapse when this process was blocked (Burbea *et al*., 2002). It was later observed in mammalian AMPARs when the GluA1 subunit was ubiquitinated following application of the agonist AMPA (Schwarz, Hall and Patrick, 2010). It has now been elucidated that all four AMPAR subunits can be ubiquitinated upon AMPA stimulation (Widagdo *et al*., 2015).

Ubiquitination involves the addition of a ubiquitin moiety to a lysine residue on a protein substrate via an isopeptide bond (Pickart, 2004). The process is catalysed by a series of enzymatic reactions involving an activating enzyme (E1), a conjugating enzyme (E2) and a ligase (E3) (Mabb and Ehlers, 2010). The locus of this process is widely contested with some arguing in favour of ubiquitination at the post-synaptic membrane to trigger AMPAR internalisation (Burbea *et al*., 2002; Schwarz, Hall and Patrick, 2010) and others arguing in favour of ubiquitination in EEs to direct receptors towards a path of degradation (Lussier *et al*., 2011; Widagdo *et al*., 2015). Furthermore, the fate of ubiquitinated AMPARs can depend on the length of the ubiquitin chain. Indeed, mono- and short-chain ubiquitination often lead to degradation via the lysosome, whereas polyubiquitination targets receptors for proteasomal degradation (Claque and Urbé, 2010).

In the ubiquitin proteasome system (UPS), polyubiquitinated proteins are degraded by the 26S proteasome into small peptides and amino acids (Goo, Scudder and Patrick, 2015). Zhang and colleagues were the first to establish that turnover of AMPARs was regulated by the UPS, by demonstrating degradation of AMPAR subunits was blocked by inhibitors of the proteasome (Zhang *et al*., 2009). This was backed up in a follow-up study that demonstrated the involvement of the E3 ligase neural precursor cell expressed, developmentally down-regulated 4-1 (Nedd4-1) (Lin *et al*., 2011).

Trafficking of AMPARs to the lysosome was first demonstrated following bath application of the agonist AMPA, where their subsequent degradation was blocked by lysosomal inhibition (Ehlers, 2000). Nedd4-1 has once again been established as the E3 ligase involved in AMPAR ubiquitination and targets these receptors for degradation via the lysosome (Schwarz, Hall and Patrick, 2010). The sorting of internalised AMPARs to the lysosome is a key determinant in LTD induction, with Fernández-Monreal and colleagues (2012) demonstrating lysosomal GluA1 degradation in response

to LTD induction. Indeed, expression of a dominant negative Rab7, which regulates trafficking to the lysosome, significantly reduced LTD expression (Fernández-Monreal *et al*., 2012). Consistent with this, the same authors demonstrated that dephosphorylation of GluA1 on Ser-845 is correlated with AMPAR lysosomal degradation, and GluA1 S845A phosphomutant mice exhibit altered LTD and constitutive AMPAR degradation by the lysosome (He *et al*., 2009). However, the role of ubiquitin in LTD-mediated AMPAR lysosomal targeting is still undetermined.

As well as ubiquitin, several proteins are thought to interact with AMPARs to control their degradation including cortactin, whose putative role in directing GluA2-containing AMPARs away from lysosomes will be discussed below.

2.3 CORTACTIN

2.3.1 Structure, homology and expression

Initially discovered as a substrate of Src kinase in chicken embryo fibroblasts (Kanner *et al*., 1990) cortactin complementary deoxyribonucleic acid (cDNA) was subsequently cloned to reveal a novel protein with a unique structure consisting of several domains (Wu *et al*., 1991) (figure 2-4).

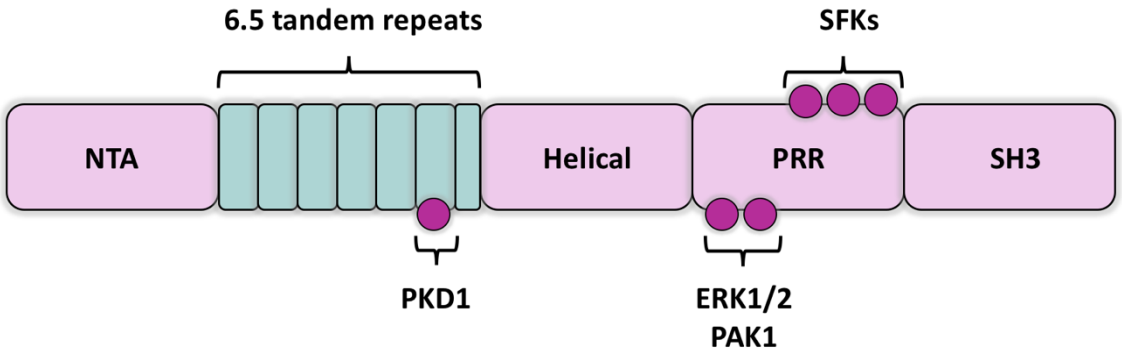

Figure 2-4 Schematic of cortactin structure and PTMs.

Domains are represented by rectangles and PTMs shown as circles. Cortactin consists of an N-terminal acidic (NTA) domain at its N-terminus (Weed et al*., 2000) followed by six tandem repeats of 37 amino acids and one incomplete repeat 20 residues in length (Sparks* et al*., 1996). These repeats are followed by an α-helical domain, a proline-rich region (PRR) and a Src homology 3 (SH3) domain at the distal C-terminus (Weed and Parsons, 2001).*

Cortactin genes have now been cloned from a number of organisms including human (Schuuring *et al*., 1993), mouse (Zhan *et al*., 1993) and rat (Ohoka and Takai, 1998). These all demonstrate structural similarities, with the SH3 domain showing the greatest degree of sequence homology suggesting it binds partners and performs similar functions preserved across a wide evolutionary spectrum (Weed and Parsons, 2001). Human cortactin is encoded by the *CTTN* gene (formerly called *EMS1*) on chromosome 11 and is ubiquitously expressed in nearly all mammalian tissues (Miglarese *et al*., 1994; Du *et al*., 1998). An exception to this is in hematopoietic cells, where its homologue hematopoietic cell-specific Lyn substrate 1 (HS1) is expressed, which shares structural and functional similarities to cortactin (Kitamura *et al*., 1989).

2.3.2 Post-translational modifications

The PRR of cortactin is abundant in tyrosine, serine and threonine residues, which serve to diversify its cellular functions by altering its biochemical properties (Lua and Low, 2005). Indeed, cortactin is phosphorylated in response to a wide range of stimuli during physiological, pathogenic and pharmacological conditions including: bacterial invasion (Dehio, Prévost and Sansonetti, 1995; Fawaz *et al*., 1997); epidermal growth factor receptor activation (Maa *et al*., 1992; Campbell, Sutherland and Daly, 1999); platelet-derived growth factor receptor activation (Kim and Wong, 1998); integrin-mediated cell adhesion (Vuori and Ruoslahti, 1995) and fibroblast growth factor receptor activation (Liu, Huang and Zhan, 1999). Cortactin is a target for phosphorylation by a range of kinases including: Src family kinases (SFKs) on tyrosine residues 421, 466 and 482 (Huang *et al*., 1997a; Huang *et al*., 1998); extracellular signal regulated kinase (Erk) and p21-activated kinase (PAK) at serine residues 405 and 418 (Campbell, Sutherland and Daly, 1999; Vidal *et al*., 2002) and protein kinase D (PKD) on serine 298 (Eiseler *et al*., 2010) (figure 2-4). Mass spectrometry has revealed additional phosphorylation sites whose physiological relevance remain unknown at present (Martin *et al*., 2006).

A vast array of biochemical studies point to a major role of cortactin in regulating cortical actin remodelling and organisation. Actin assembly, the synthesis of F-actin from globular actin (G-actin), underlies multiple cellular processes. A major mechanism for the nucleation of actin polymerisation is catalysed by the actin-related protein (Arp)2/3 complex, which is activated by nucleation promoting factors including Neuronal Wiskott Aldrich Syndrome protein (N-WASP) (Kreishman-Deltrick & Rosen, 2002; Welch and Mullins, 2002). Consistent with a role for cortactin in actin assembly, it can directly bind to F-actin (Wu and Parsons, 1993) and stabilise newly generated filaments (Weaver *et al*., 2001), with the fourth cortactin repeats being absolutely necessary for this

interaction and the third and fifth repeats required for efficient binding (Weed *et al*., 2000). Furthermore, cortactin can bind to the Arp2/3 complex to stimulate actin nucleation independently, mediated through a three amino acid motif in the NTA (Weed *et al*., 2000), as well as in conjunction with N-WASP, through a synergistic interaction with the C-terminal verprolin-homology domain, cofilin-homology, and acidic region (VCA) domain (Weaver *et al*., 2001). Src and Erk phosphorylation work together to regulate cortactin-mediated actin remodelling. Indeed, upon phosphorylation by Erk, the cortactin SH3 domain is liberated from intramolecular interaction with the PRR and able to activate N-WASP and promote actin polymerisation (Martinez-Quiles *et al*., 2004). Conversely, Src phosphorylation of cortactin inhibits its ability to bind to and activate N-WASP (Martinez-Quiles *et al*., 2004), decreases its F-actin-binding capacity (Huang *et al*., 1997a) and promotes its cleavage by calpain (Huang *et al*., 1997b), all of which terminate actin polymerisation. The Erk/Src phosphorylation of cortactin is consequently known as an on-off switch for actin assembly.

2.3.3 Cortactin and endocytosis

Many studies have shown that cortactin can regulate both CME and CIE in non-neuronal cells (Kirkbride *et al*., 2011). It is understood that the polymerisation of actin provides the force for the deformation and movement of the membrane at different stages of endocytosis (Kaksonen, Toret and Drubin, 2006). Indeed, actin co-localises with sites of CME in neuronal synapses (Shupliakov *et al*., 2002) and in mammalian cells, bursts of actin appear at 80% of internalising clathrin-coated pits on the surface membrane (Merrifield *et al*., 2002). It appears that bursts of actin polymerisation are at their peak during vesicle scission (Merrifield, Perrais and Zenisek, 2005). Consistent with this, several studies have demonstrated that actin is not required for the formation of clathrin-coated pits but instead is necessary for their scission from the membrane (Merrifield, Perrais and Zenisek, 2005; Yarar, Waterman-Storer and Schmid, 2005). Being an actin binding protein, cortactin function is also important in mediating these events. CIE is reliant on PAK phosphorylation of cortactin to increase its association with N-WASP (Grassart *et al*., 2010) whereas CME relies on a Src-mediated interaction of cortactin with dynamin-2 (Cao *et al*., 2009). Furthermore, co-localisation of cortactin with both clathrin and dynamin-2 puncta has been witnessed on the cell surface, and peak cortactin recruitment also coincides with vesicle scission (Cao *et al*., 2003; Merrifield, Perrais and Zenisek, 2005). It must be noted that not all cell types are reliant on cortactin for endocytosis, since some studies report no change in CME upon knockdown of cortactin expression (Barroso *et al*., 2006; Lai *et al*., 2009).

2.3.4 Cortactin and endosomal trafficking events

Cortactin has also demonstrated a role in endosomal trafficking events. Indeed, cortactin can associate with actin tails on endosomal vesicles to aid 'actin rocketing', the propulsive force that drives the trafficking of endosomes through the cytoplasm (Kaksonen, Peng and Rauvala, 2000). Furthermore, in non-neuronal cells cortactin is localised to tubular domains of sorting endosomes and mediates the selective recycling of the receptors back to the cell surface. Indeed, cortactin was required for the localisation of the β2-adrenergic receptor (β2AR) into specialised recycling tubules, possibly through its ability to promote Arp2/3-dependent actin polymerisation (Puthenveedu *et al*., 2010), a process which was positively modulated by Src phosphorylation of cortactin (Vistein and Puthenveedu, 2014).

Cortactin has also demonstrated a role in receptor recycling in neuronal cells. In a proteomics screen, cortactin has previously been established as an AMPAR associated protein (Klemmer, Smit and Li, 2009), however the role of this association was not explored until recently. Indeed, it has now been determined that cortactin maintains surface levels of GluA2/3 heteromers by directing receptors away from lysosomes (Parkinson *et al*., 2018). In the absence of a cortactin-GluA2 interaction, GluA2-containing AMPARs were targeted for lysosomal degradation. The interaction was negatively regulated by SFK phosphorylation of cortactin at tyrosine residues Y421 and Y466, a process that occurred downstream of NMDAR stimulation and thus a likely component of LTD expression (Parkinson *et al*., 2018).

Endosomal dysfunction is an early indicator for a number of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Schreij, Fon and Mcpherson, 2015), Niemann-Pick type C1 (D'Arcangelo *et al*., 2011; Rabenstein *et al*., 2017) and neuropathologies such as ischemia (Yuan, Liu and Hu, 2017). Critically, these endosomal deficits result in aberrant lysosomal targeting of crucial synaptic proteins, such as AMPARs, and are thought to underlie the impairments in learning and memory seen in these pathologies. The dysregulation of endosomal trafficking events in Alzheimer's disease will be discussed in more detail below.

2.4 ALZHEIMER'S DISEASE

2.4.1 Epidemiology, Aetiology and Pathology

First described by the German neuropathologist Alois Alzheimer, AD is a progressive neurodegenerative disorder characterised by cognitive impairment which typically begins with memory deficits and eventually develops to incapacitating levels (Alzheimer, 1907). It is the most common form of dementia accounting for over 50% of cases, and its prevalence increases 15-fold between the ages of 60 and 85 putting today's increasingly aged population under intense socioeconomic burden (Reitz, Brayne and Mayeux, 2011; Jurado, 2018). There are two forms of the disease, familial and sporadic, both of which are associated with different patterns of genetic epidemiology. Sporadic AD accounts for over 95% of cases and is not inherited in a Medelian fashion but instead is associated with genes that increase the risk of developing the disease. These include the ε4 allele of Apolipoprotein E (*APOE*) which is thought to account for 20-30% of AD risk (Reitz, Brayne and Mayeux, 2011). Three genes have been firmly implicated in the pathophysiology of familial AD: *APP* and the presenilin genes *PSEN1 and PSEN2*. AD-linked mutations in these genes are considered diagnostic biomarkers of the disease and lead with certainty to early-onset AD (Reitz, Brayne and Mayeux, 2011).

Two proteins make up the key pathological hallmarks of AD: Amyloid-β (Aβ), which accumulates extracellularly in diffuse and neuritic plaques; and tau, a microtubule-binding protein which is hyper-phosphorylated and deposited intracellularly as neurofibrillary tangles (Reitz, Brayne and Mayeux, 2011). These pathologies are accompanied by reactive microgliosis and a widespread loss of neurons, synapses and white matter. Although both tau and Aβ are key to the pathogenesis of AD, strong evidence from genetics and transgenic mice has implicated Aβ to be strongly associated with disruption of synaptic function (Guntupalli, Widagdo and Anggono, 2016), thus this thesis will focus on this hallmark of the disease.

2.4.2 Amyloid Precursor Protein Processing

First cloned in 1987, the amyloid precursor protein (APP) is a single-pass transmembrane protein whose precise physiological function remains one of the most vexing questions in the field (Kang *et al.*, 1987; O'Brein and Wong, 2011). The *APP* gene is located on chromosome 21 and alternatively

spliced into 3 major isoforms: the 751 and 770 amino acid forms, which are expressed ubiquitously; and the 695 amino acid form which is expressed primarily in the central nervous system (Bayer *et al*., 1999).

Newly synthesised APP is delivered from the *trans*-Golgi network to the cell surface where it is directly cleaved or re-internalised into endosomes. On the cell surface, APP undergoes proteolysis by α-secretase and then γ-secretase. γ-secretase is a multiprotein complex composed of presenilin 1 or presenilin 2, which form the catalytic core; nicastrin, a type I transmembrane glycoprotein; and Aph-I and Pen-2, two multi-pass transmembrane proteins (Bergmans and De Strooper, 2010). If re-internalised, APP is cleaved by β-secretase 1 (BACE1) and γ-secretase which results in the production of Aβ (O'Brien and Wong, 2011) (figure 2-5). A recently discovered secretase, η-secretase, can also cleave APP in the non-amyloidogenic pathway liberating sAPPη, and leaving behind membrane-bound CTFη (Willem *et al*., 2015). In turn, CTFη can be further cleaved by α- or β- secretase resulting in the extracellular release of Aη-α or Aη-β (Willem *et al*., 2015). The effects of these cleavage products on neuronal function and circuitry will be discussed in section 2.4.8.

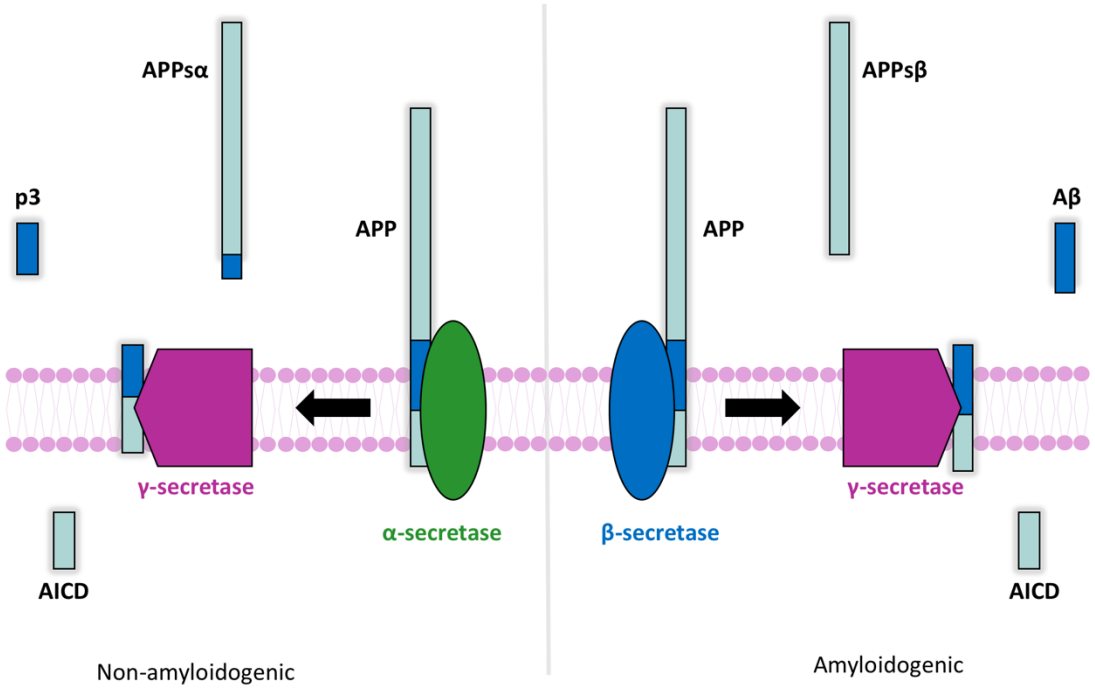

Figure 2-5 Processing of APP.

APP is cleaved by α- followed by γ-secretase in the non-amyloidogenic pathway, leading to secretion of APPsα and p3, and retention of the APP intracellular domain (AICD). In the amyloidogenic pathway, APP is cleaved by β- followed by γ-secretase, secreting the APPsβ and Aβ fragments, and retaining the AICD.

The γ-secretase cleavage of APP is variable and thus three forms of Aβ are produced: $A\beta_{38}$, $A\beta_{40}$ and $A\beta_{42}$. The precise site of γ-secretase cleavage has an important influence on the resulting

pathogenicity because only the $A\beta_{42}$ peptide has the strong propensity to oligomerise *in vivo* (Haass and Selkoe, 2007). Indeed, all AD-causing mutations in *PSEN1* and *PSEN2* and some in *APP* enhance the production of the $A\beta_{42}$ peptide (Suzuki *et al*., 1994; Scheuner *et al*., 1996). Once formed, Aβ can assume a number of conformation states including monomers, soluble oligomers, protofibrils and fibrils, which aggregate to form plaques (Koffie, Hyman and Spire-Jones, 2011). It was initially understood that aggregated plaques were the pathological form of Aβ, however it is now believed that Aβ oligomers hold greater toxicity (Selkoe, 2008). Indeed, levels of soluble Aβ oligomers correlate more strongly with cognitive deficits in AD patients than plaque counts (Näslund *et al*., 2000) and APP transgenic mice with increased soluble Aβ in the brain display notable cognitive impairments before the onset of plaque deposition (Mucke *et al*., 2000). Furthermore, Aβ oligomers are significantly correlated with synaptic deficits both *in vitro* and *in vivo* – namely, the inhibition of LTP (Hu *et al*., 2008; Shanker *et al*., 2008) and facilitation of LTD (Kim *et al*., 2001; Li *et al*., 2009).

2.4.3 Models of Alzheimer's Disease

Experimental models of AD are vital to better understand the pathophysiology of the disorder and for testing novel therapeutics. However, success in AD clinical trials has been limited thus far due to poor translation of successful preclinical testing in animal models into efficacious treatments (Banik *et al*., 2015). The most commonly used animal models are transgenic mice that overexpress human genes associated with familial AD (figure 2-6) and mimic a plethora of pathologies, most notably the formation of Aβ plaques (Drummond and Wisniewski, 2017). The first transgenic mouse model developed in 1995 was the PDAPP mouse, which expressed the Indiana mutation (APP_{V717F}) and resulted in vast over-expression of APP (Games *et al*., 1995). The Tg2576 mouse was generated soon after, expressing human APP with the double Swedish mutation ($APP_{K670N/M671L}$) and also resulting in dramatic APP over-expression (Hsiao *et al*., 1996). It was later discovered that expressing numerous familial AD mutations at once resulted in more severe pathology, thus mice such as the J20 model were developed, which expresses both the Swedish and Indiana mutations (Mucke *et al*., 2000). The major limitation with these models was that they only developed amyloid accumulation; indeed, none developed the neurofibrillary tangles that also define the disease (Karch and Goate, 2015). The expression of both plaques and tangles has proved troublesome, but the 3xTg transgenic mouse expressing the mutations $APP_{K670N/M671L}$, $PSEN1_{M146V}$ and $MAPT_{P301L}$ is the most widely used model of the two pathological hallmarks (Oddo *et al*., 2003).

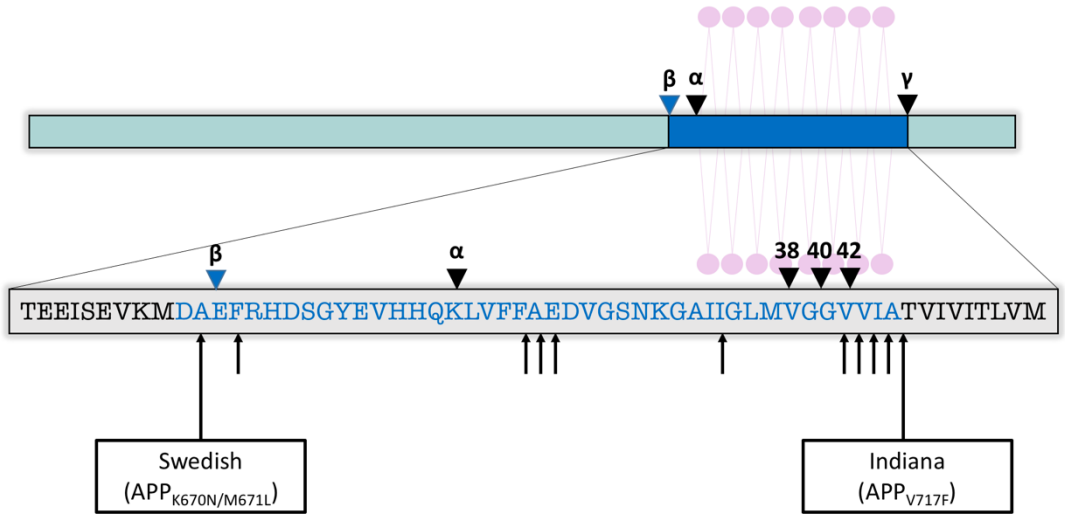

Figure 2-6 Familial APP mutations.

Black arrows mark the most common mutations leading to familial AD. The length of the resultant Aß fragment is indicated by numbers above the cleavage sites for γ-secretase.

Brain tissue *in vitro* provides an additional platform to model AD. Neuronal cell cultures afford an easily manipulated and controlled environment suitable for testing therapeutics (Nepovimova *et al*., 2014) and for revealing causative mechanisms of disease states at a molecular and cellular level (Akhter, Sanphui and Biswas, 2014). Application of Aβ to these cultures can replicate its actions in AD, as well as the use of deoxyribonucleic acid (DNA) transfection protocols or viral infection of APP constructs (Hsieh *et al*., 2006). One of the major drawbacks of neuronal cell cultures is their failure to mimic the *in vivo* physiology of the brain, a problem that can be partially overcome by the use of brain slices that reside in their natural physiological environment (Gong *et al*., 2001).

2.4.4 Synaptic dysfunction in Alzheimer's disease

Malformations in synapses from AD brain tissue were first described more than five decades ago (Gonatas, Anderson and Evangelista, 1967) and since then much evidence has reinforced the notion that AD is primarily a disorder of synaptic failure, being increasingly referred to as a 'synaptopathy'. Indeed, it is now believed that synapse loss demonstrates a strong association with cognitive severity and may provide the best correlate of dementia (Sze *et al*., 1997; Terry *et al*., 1991).

Molecular biomarkers of active synapses can be used as early indicators of synaptic damage such as synaptic proteins, which make ideal surrogates for markers of synapses *in vivo* (Davidsson *et al*.,

1996). Indeed, a loss of synaptophysin immunoreactivity of up to 25% has been established in frontal cortices of mild AD cases, with synaptotagmin and growth-associated protein 43 (GAP43) showing progressive decrement in more advanced cases (Masliah *et al.,*1989; Masliah *et al*., 2001). The pre-synaptic vesicle protein Rab 3A and postsynaptic protein synaptopodin have also demonstrated significant down-regulation in AD brain specimens (Reddy *et al*., 2005). As many as 44% of synaptic boutons are lost in AD patients compared to age-matched controls (Scheff *et al*., 2006), with a loss of synaptic contacts most readily documented in both the neocortex and the hippocampus (Bertoni-Freddari *et al*., 1990; DeKosky and Scheff, 1990; Scheff and Price, 1998; Paula-Barbosa *et al*., 2009).

Changes in synapse number are accompanied by electrophysiological phenotypes. Indeed, a multitude of studies have demonstrated that oligomeric Aβ inhibits the induction of LTP both *in vivo* (Cullen *et al*., 1997; Walsh *et al*., 2002) and *in vitro* (Wang *et al*., 2002; Shanker *et al*., 2007, Shanker *et al*., 2008), whilst the induction of LTD is facilitated (Hsieh *et al*., 2006; Shanker *et al*., 2008; Li *et al*., 2009). Since LTP is the cellular correlate of learning and memory, these phenomena are accompanied by significant deficits in cognition. In accord with this, intra-cerebral injection of synthetic or naturally secreted Aβ incurs detrimental effects on the behaviour of rats, as measured in the Morris water maze (Chen, Wright and Barnes, 1996; Delobette, Privat and Maurice, 1997) and the alternating lever cyclic ratio test (O'Hare *et al*., 1999; Cleary *et al*., 2004). APP transgenic mouse models also demonstrate a negative correlation between accumulation of Aβ in the hippocampus and spatial memory in the Morris water maze (Hsiao *et al*., 1996; Chen *et al*., 2000; Mucke *et al*., 2000) and neutralisation of soluble oligomeric Aβ with anti-Aβ antibodies can reverse these behavioural deficits (Dodart *et al*., 2002; Kotilinek *et al*., 2002).

It is important to note that a small but growing body of evidence suggests that the negative effects of Aβ on LTP are concentration dependent, since low concentrations in the picomolar range actually facilitate LTP (Puzzo *et al*., 2008; Puzzo *et al*., 2011). These observations suggest that endogenous levels of Aβ in the healthy nervous system are required for synaptic plasticity but that accumulation of this protein in disease states induces a toxic function. These opposing functions mean Aβ homeostasis at the synapse must be intricately balanced.

How Aβ can facilitate the induction of LTD has begun to be elucidated in emerging evidence suggesting that toxic levels of Aβ result in the aberrant activation of extrasynaptic NMDARs (eNMDARs) (Li *et al*., 2009; Li *et al*., 2011). It has previously been demonstrated that eNMDARs can trigger *de novo* LTD (Massey *et al*., 2004). Consistent with this, Aβ oligomers facilitate LTD in CA1 cells through activation of these GluN2B-containing eNMDARs (Li *et al*., 2009). This activation results, at least in part, from the inhibition of glutamate uptake and resultant 'spillover' to

extrasynaptic sites, since LTD facilitation is blocked by extracellular glutamate scavengers (Li *et al*., 2009). Indeed, previous findings demonstrate increased extracellular glutamate levels in response to soluble Aβ oligomers (O'Shea *et al*., 2008) and expression levels of the excitatory amino acid transporters 1 and 2 (EAAT1 and EAAT2) are altered in AD subjects (Jacob *et al*., 2007). In addition, oligomeric Aβ triggers aberrant astrocyte glutamate release (Talantova *et al*., 2013). Ca^{2+} influx through the GluN2B-containing eNMDARs results in the activation of protein phosphatases, suggesting Aβ oligomers shift the activation of NMDAR-dependent signalling cascades toward pathways involved in the induction of LTD (Knobloch *et al*., 2007; Miñano-Molina *et al*., 2011). Furthermore, excitotoxicty can be triggered by activation of NMDARs that contain GluN2B subunits (Zhou and Baudry, 2006a; Liu *et al*., 2007) providing another mechanism by which this additional Ca^{2+} influx may contribute to neurodegeneration.

2.4.5 AMPAR trafficking in Alzheimer's disease

2.4.5.1 AMPAR endocytosis

Previously described in section 2.1.4, one of the key mechanisms controlling synaptic plasticity expression is the number of AMPARs at the synapse which requires a tight balance between their biosynthesis, trafficking and degradation. It is the loss of control of this tight balance that leads to synaptic depression and deficits in learning and memory in AD (Anggono and Huganir, 2012). Indeed, a host of literature has demonstrated a role for Aβ in promoting the endocytosis of AMPARs (Hsieh *et al*., 2006; Liu *et al*., 2010; Zhao *et al*., 2010; Miñano-Molina *et al*., 2011; Alfonso *et al*., 2014). Furthermore, the blockade of endocytosis prevents Aβ-induced synaptic depression (Hsieh *et al*., 2006). This synaptic depression is dependent on NMDAR activity (Kamenetz *et al*., 2003; Shankar *et al*., 2007), suggesting it shares common signalling pathways with LTD; namely changes to posttranslational modifications and AMPAR interacting proteins.

2.4.5.2 Mechanisms of AMPAR internalisation

Among others, one consequence of Ca^{2+} influx through the GluN2B-containing eNMDARs is the activation of the protein phosphatases calcineurin and PP1 (Knobloch *et al*., 2007; Miñano-Molina *et*

al., 2011). Activation of calcineurin results in dephosphorylation of target proteins and enhances AMPAR endocytosis. Indeed, $APP_{SWE,IND}$ transgenic mice exhibit reduced Ser-845 phosphorylation and reduced surface AMPAR expression (Miñano-Molina *et al*., 2011). In the presence of the selective calcineurin inhibitor FK506 however, oligomer-induced GluA2 surface loss is prevented (Zhao *et al*., 2009).

As mentioned in section 2.2.4.3, phosphorylation of GluA2 at Ser-880 weakens its interaction with GRIP, allowing PICK1 to bind and AMPARs to be internalised (Seidenman *et al*., 2003). In hippocampal neuronal cultures it has been observed that oligomeric Aβ can highjack this process to increase Ser-880 phosphorylation of GluA2 and reduce AMPAR surface expression (Liu *et al*., 2010). Also consistent with a role for PICK1 in Aβ-mediated pathogenesis, in cultured neurons prepared from mice in which PICK1 has been genetically ablated, Aβ failed to reduce surface levels of the GluA2 receptor subunit. Furthermore, application of a small molecule, BIO922, which targets the interaction between GluA2 and PICK1 blocked Aβ-induced synaptic depression (Alfonso *et al*., 2014).

Aβ has also been shown to re-distribute CaMKII away from synaptic sites in APP_{SWE} transgenic mice and in Aβ-treated cultures (Gu, Liu and Yan, 2008). Previously described in section 2.1.4, CaMKII can potentiate excitatory transmission via phosphorylation of both GluA1 at Ser-831 to increase single channel conductance and stargazin to aid the binding of AMPARs to the slot protein PSD-95. Consistent with this role, altered CaMKII localisation reduced AMPAR-mediated synaptic responses and resulted in a loss of GluA1 surface expression in APP_{SWE} transgenic mice and Aβ-treated cultures respectively (Gu, Liu and Yan, 2008). Aβ can also destabilise synapses by affecting PSD-95 directly. Indeed, synaptic expression of PSD-95 is markedly reduced in post-mortem brains of AD patients (Gylys *et al*., 2004; Proctor, Coulson and Dodd, 2010; Sultana, Banks and Butterfield; 2010) and in neurons from AD transgenic mice, which is concomitant to surface AMPAR removal (Almeida *et al*., 2005).

Aβ oligomers have been shown to preferentially target different AMPAR subunits, with a selection of literature demonstrating a preference for the GluA2 subunit. Indeed, the expression of GluA2 receptor subunits has defined AMPAR removal in numerous studies: Hsieh *et al* (2006) demonstrated that a GluA2 mutant unable to undergo AP2-dependent endocytosis blocked Aβ-induced AMPAR removal; Zhao *et al* (2009), with the use of co-immunoprecipitation and photoreactive amino acid cross-linking, found that oligomeric assemblies of Aβ preferentially affect GluA2-containing AMPARs; and both Liu *et al* (2010) and Alfonso *et al* (2014) discovered Aβ to trigger PICK1-induced surface removal of the GluA2 receptor subunit. However, a more recent study suggests that the effects of Aβ on synapse impairment depends on the subunit GluA3 (Reinders *et*

al., 2016). Hippocampal neurons lacking GluA3 didn't demonstrate Aβ-mediated synaptic depression, GluA3-deficient brain slices were resistant to Aβ-induced LTP deficits and GluA3 deficient APP/PS1 transgenic mice did not display spine loss or memory impairment (Reinders *et al*., 2016). Consistent with this, a recent study screening for gene-expression profiles associated with mild cognitive impairment, a transitional stage between aging and AD, highlighted GluA3 in having a strong negative correlation with cognitive performance (Berchtold *et al*., 2014). Although seemingly conflicting results, considering most GluA3 subunits exist as GluA2/3 AMPARs (Wenthold *et al*., 1996) a model arises in which Aβ oligomers favourably trigger the endocytosis of these heteromers from the synaptic surface or affect their endosomal recycling.

Whilst in general Aβ facilitates AMPAR internalisation and synaptic depression, there is evidence that in some cases Aβ can increase GluA1-containing AMPARs at the surface. Indeed, when postsynaptic density-rich fractions of hippocampi from human AD patients were examined, an increase in GluA1 expression was reported when compared to control patients (Marcello *et al*., 2012). It has also been reported that both APP/PS1 transgenic mice and hippocampal neuronal cultures treated with oligomerised Aβ show aberrant CP-AMPAR expression and insertion mediated by protein kinase A (PKA) phosphorylation of GluA1 on Ser-845 (Megill *et al*., 2015; Whitcomb *et al*., 2015). The mechanisms by which CP-AMPARs contribute to neurodegeneration in AD have not been elucidated, but since under physiological conditions CP-AMPARs appear only transiently in response to synaptic activity (Plant *et al*., 2006) it is likely that aberrant activation of these receptors in the disease state leads to excitotoxicity, a pivotal catalyst for the onset of pathology.

2.4.5.3 AMPAR degradation

With a multitude of studies describing the loss of AMPARs from the synaptic surface, whether or not this is accompanied by subunit degradation has been explored as well as the underlying molecular mechanisms that might be involved.

There exists conflicting evidence from post-mortem examinations of AD brain tissue and neuronal cell culture models as to whether or not GluA1 and GluA2 receptor subunits show reduced expression levels in the disease state. Indeed, there is evidence for both GluA2 (Carter *et al*., 2004; Resende *et al*., 2007) and GluA1 subunit degradation (Yasuda *et al*., 1995), whereas other studies indicate no change in subunit expression levels (Pellegrini-Giampietro, Bennett and Zukin, 1994; Rodrigues *et al*., 2016).

Previously described in section 2.2.4.4 ubiquitination is a major signal for the sorting of cargo to the proteosome and the late endosome, a process that has recently been shown to be highjacked in AD (Rodrigues *et al*., 2016; Guntupalli *et al*., 2017; Zhang *et al*., 2018). It has been demonstrated that incubation of hippocampal neurons with Aβ results in increased expression of the E3 ligase Nedd4-1 (Zhang *et al*., 2018) and its recruitment to dendritic spines (Rodrigues *et al*., 2016). As a result, ubiquitination of the GluA1 subunit is increased (Rodrigues *et al*., 2016; Guntupalli *et al*., 2017; Zhang *et al*., 2018) leading to reduced AMPAR surface expression and a reduction in total AMPAR levels (Zhang *et al*., 2018).

As discussed previously, GluA2 can be tyrosine phosphorylated on Tyr-876 by SFKs which regulates AMPAR surface expression and synaptic targeting of GluA2 (Chung *et al*., 2000; Hayashi and Huganir, 2004). There is some evidence for increased activity of these kinases in AD, which suggests this process could be hi-jacked in the disease state.

2.4.6 Src family kinases

The Src kinase family is a family of non-receptor tyrosine kinases that contains nine members including Src and Fyn. They consist of six conserved domains: a myristoylated N-terminal segment, an SH2 and SH3 domain, a linker region, a tyrosine kinase domain and a C-terminal tail (Parsons and Parsons, 2004). A number of substrates have been identified for this kinase family which are modified through phosphorylation of tyrosine residues (Amanchy *et al*., 2008). SFKs participate in signalling pathways that control a diverse spectrum of cellular functions, thus their dysregulation can contribute to the pathophysiology of numerous disease states and manifestations.

Among these, emerging evidence has identified a link between aberrant SFK activity and AD, particularly the kinase Fyn which is located at the PSD of glutamatergic synapses and therefore able to modulate the effects of Aβ on synaptic function (Chin *et al*., 2005). Indeed, *in vitro* application of Aβ has been shown to increase tyrosine phosphorylation of proteins in various preparations (Luo *et al*., 1995; Bamberger *et al*., 2003; Grace and Busciglio, 2003) and histologic analysis of post-mortem brain sections from AD patients has revealed enhanced Fyn immunoreactivity (Shirazi and Wood, 1993). The first evidence for a direct involvement of Fyn in AD came in 1998 when slices from Fyn knockout mice were spared of Aβ-mediated toxicity (Lambert *et al*., 1998). Since then APP transgenic mouse models have demonstrated that Fyn overexpression both accelerates the onset of cognitive impairment and promotes Aβ-induced synaptotoxicity, a phenomenon which can be rescued by Fyn

ablation (Chin *et al*., 2004, Chin *et al*., 2005). Despite much evidence for aberrant Fyn activity, the signalling pathway by which Aβ activates Fyn was not elucidated until much later.

When applied to neuronal cultures, Aβ oligomers show a binding pattern suggesting the presence of a receptor on the cell surface (Lacor *et al*., 2007; Laurén *et al*., 2009). Whilst many receptors have been proposed (Jarosz-Griffiths *et al*., 2015), the cellular prion protein (PrPc), a widely expressed protein highly abundant in the central nervous system, is a strong candidate (Westergard, Christensen and Harris, 2007). Indeed, PrPc interacting with Aβ has been regularly established in human AD brain homogenates (Barry *et al*., 2011; Um *et al*., 2012; Haas *et al*., 2015) and removing PrPc expression prevents impairments in LTP, spatial memory and synapse loss in AD transgenic mice and Aβ-exposed cultures (Laurén *et al*., 2009; Gimbel *et al*., 2010; Barry *et al*., 2011). It is thus believed that Aβ may activate Fyn kinase via this cell surface receptor. In accord with this, exposing cultured neurons to Aβ results in increased levels of phosphorylated Y416-Fyn in a PrPc-dependent manner (Um *et al*., 2012). Furthermore, both PrPc and Fyn were required for Aβ-induced spine loss (Um *et al*., 2012). This signalling cascade is thus a likely pathway for conducting downstream pathogenesis.

2.4.7 Neuronal circuit pathogenesis

Over the past few years, a clear clinical insight that has emerged is that of pathologically disrupted cortico-hippocampal circuits in AD, in which a disequilibrium in neuronal excitation and inhibition manifests as hyperactivity in pre-clinical stages (Harris *et al*., 2020). Hyperactivity of the hippocampus is of interest because it predicts a greater extent and rate of cognitive decline (Miller *et al*., 2007), and is significantly related to the deposition of Aβ in the brain (Leal *et al*., 2017). Targeting this hyperactivity with the antiepileptic levetiracetam resulted in normalisation of hippocampal activity levels and improved memory performance in mildly impaired individuals with AD (Bakker *et al*., 2012; Bakker *et al*., 2015). These findings suggest that pathological hippocampal activation is related to Aβ pathology and contributes to memory impairment.

Much evidence indicates that soluble Aβ oligomers are key players in driving neuronal hyperactivity in AD. Indeed, administration of Aβ oligomers in the absence of plaque pathology can induce neuronal hyperexcitability in the hippocampus (Busche *et al*., 2012) and cortex (Keskin *et al*., 2017). The mechanisms by which Aβ exerts its effects remain an area of active research (Harris *et al*., 2020) but include a reduction in GABAergic terminals and activity (Busche *et al*., 2015; Garcia-Marin *et al*., 2009; Sun *et al*., 2012) as well as impaired glutamate homeostasis (Zott *et al*., 2019).

At first, Aβ-mediated increases in net excitatory drive, impairment of LTP and facilitation of LTD might seem paradoxical, however, this might be explained by Aβ-mediated modulation of synaptic glutamate mechanisms (Harris *et al*., 2020) including a shift in excitatory/inhibitory balance (by augmenting presynaptic glutamate release or blocking glutamate re-uptake) (Lei *et al*., 2016; Wang *et al*., 2017). This impaired synaptic glutamate handling might subsequently lead to increased synaptic activation of NMDARs and their desensitisation, followed by internalisation of NMDARs and AMPARs (as shown to be promoted by Aβ) (Hsieh *et al*., 2006; Snyder *et al*., 2005; Um *et al*., 2012) and activation of extra-synaptic NMDA and metabotropic glutamate receptors because of glutamate spill over (implicated in Aβ-mediated facilitation of LTD) (Hsieh *et al*., 2006; Li *et al*., 2009). In addition, Aβ-induced activation of the calcineurin (Wu *et al*., 2010), as a result of increases in Ca^{2+} influx, might be expected to result in NMDAR and AMPAR endocytosis and lead to impairment of LTP and facilitation of LTD (Hsieh *et al*., 2006; Snyder *et al*., 2005).

2.4.8 APP and other peptides

In addition to the effects of the hallmark peptides in AD on neuronal function, evidence has surprisingly emerged suggesting that the APP processing pathway comprises several components able to modulate neuronal function and circuit activity. Notably, APP695 associates with GluN2A and GluN2B-containing NMDARs and enhances their cell-surface delivery (Cousins *et al*., 2009), suggesting that APP may actively regulate postsynaptic excitatory processes. Interestingly, APP related proteins, amyloid precursor- like proteins 1 and 2 (APLP1/APLP2) behave similarly to APP in that they also associate with NMDARs to regulate surface expression (Cousins, Dai and Stephenson, 2015). Furthermore, APP affects hippocampal GABAergic transmission (Wang *et al*., 2014) through presynaptic modulation of Cav1.2 L-type Ca^{2+} channels (Yang *et al*., 2009). APP binds to $GABA_B$ receptors, thereby enhancing receptor trafficking and cell-surface expression and promoting presynaptic inhibition (Dinamarca et al., 2019). These findings implicate APP as contributing to the regulation of the excitatory/inhibitory balance in the brain.

Several of the APP cleavage products have also been recently implicated in modulating excitatory and inhibitory processes. Indeed, all three sAPP cleavage forms, sAPPα, sAPPβ and sAPPη, bind to the $GABA_B$ receptor subunit 1a, and sAPPα decreases synaptic vesicle recycling, reduces the frequency of excitatory and inhibitory postsynaptic currents in cultured hippocampal neurons and enhances short-term facilitation in acute mouse hippocampal slices (Rice *et al*., 2019). As such, sAPP reduces the Pr of synaptic vesicles to modulate synaptic transmission and plasticity (Rice *et al*., 2019). Notably, when Aη-α, a recently discovered fragment derived from η-secretase APP processing, was applied to hippocampal slices ex vivo, LTP was lowered (Willem *et al*., 2015).

Furthermore, in vivo single-cell two-photon Ca^{2+} imaging showed that hippocampal neuronal activity was attenuated by Aη-α, providing further evidence for the modulation of neuronal activity by APP cleavage products (Willem *et al*., 2015).

2.4.9 Emerging therapeutic approaches in AD

There is a huge need for therapies to prevent and slow the progression of AD, however, the major challenge in AD drug development is the lack of understanding about the mechanisms underlying AD pathogenesis and pathophysiology (Cao *et al*., 2018). To date, only symptomatic treatments exist for the disease: three cholinesterase inhibitors for the treatment of mild to moderate AD and memantine, an NMDAR non-competitive antagonist, for the treatment of moderate to severe AD (Yiannopoulou & Papageorgiou, 2012).

Several emerging therapies targeted at amyloid pathology have been the focus of development of AD strategies. These include the development of BACE1 inhibitors, which have mostly failed to survive phase II/II studies (Cao *et al*., 2018). However, despite the disappointing results from current clinical trials of BACE1 inhibitors, a recent study demonstrated that conditional knockout of BACE1 was capable of completely reversing pre-formed amyloid deposition and improving cognitive function in a mouse model with 5× FAD transgenic background, suggesting sequential and gradual inhibition of BACE1 could be beneficial for AD patients (Hu *et al*., 2018). Substantial effort has also been invested into developing small-molecule inhibitors of γ-secretase for AD therapies. Both non-selective and selective γ-secretase inhibitors have been tested in clinical trials, but most have been discontinued because of lack of efficacy or adverse side effects (Cao *et al*., 2018).

As well as strategies to reduce Aβ generation, accelerating Aβ clearance via immunotherapy with active and passive vaccines against Aβ has also been utilised as a therapeutic approach in AD with Gantenerumab and aducanumab targeted at both soluble and aggregated Aβ species currently in Phase II trials (Cao *et al*., 2018).

Other therapeutic strategies include those targeted at tau and ApoE, neuroprotective strategies, symptomatic and cognitive enhancers and interventions for AD prevention (Cao *et al*., 2018). As new insights into AD pathogenesis and progression are gained, developing or repurposing drugs with the capacity to target different aspects of the disease pathogenesis at once become promising in AD therapies (Cao *et al*., 2018).

3 AIMS AND HYPOTHESIS

To summarise, dysregulation of AMPAR trafficking contributes to synaptic dysfunction and cognitive deficits in AD. Thus, identifying the molecular mechanisms underlying aberrant AMPAR trafficking, and the proteins that might regulate them are of particular interest in order to recognise therapeutic targets for novel drug design. AMPARs are lost from the surface in AD due to impaired recycling mechanisms and there is evidence for increased activation of the SFK Fyn in the disease. Tyrosine phosphorylation of cortactin by SFKs causes a dissociation of cortactin from GluA2, resulting in lysosomal targeting of this receptor subunit. In this project three main aims were identified and addressed as follows:

- Investigate the degradation of the GluA1 and GluA2 AMPAR subunits in three different models of AD.
    - Western blotting or immunocytochemistry for GluA1 and GluA2 receptor subunits on cultured hippocampal neurons with APP_{WT} overexpression, cultured cortical neurons with APP_{SWE} expression or J20 mouse models.

- Elucidate the effect of APP_{WT} overexpression on GluA1 and GluA2 lysosomal targeting.
    - Immunocytochemistry on cultured hippocampal neurons overexpressing APP_{WT}.

- Examine the effect of APP_{SWE} on cortactin tyrosine phosphorylation.
    - Western blotting for phospho-Y466-cortactin and phospho-Y421-cortactin on cultured cortical neurons expressing APP_{SWE}.

We hypothesised that:

- Expression levels of GluA1 and GluA2 would be reduced in all three models of amyloidopathy due to their degradation in response to Aβ.

- APP_{WT} overexpression would lead to an increase in GluA1 and GluA2 lysosomal targeting.

- APP_{SWE} expression would lead to an increase in cortactin tyrosine phosphorylation.

4 METHODS

4.1 MATERIALS

4.1.1 Antibodies

The primary antibodies used are given in table 4-1.

Table 4-1 Primary antibodies used for Western blotting (WB) and immunocytochemistry (ICC)

Antibody	Species	Supplier	Dilution	Type	Product code
Anti-APP (APP_{695}, APP_{751}, APP_{770})	Rabbit	Sigma-Aldrich	1:5000 (WB)	IgG	A8967
Anti-cortactin	Mouse	Millipore	1:1000 (WB)	IgG	05-180
Anti-GluA1	Rabbit	Upstate	1:1000 (WB)	IgG	04-855
Anti-GluA1	Mouse	Millipore	1:110 (ICC, total) 1:30 (ICC, surface)	IgG	MAB2263
Anti-GluA2	Mouse	BD Pharmingen	1:500 (WB) 1:110 (ICC, total) 1:30 (ICC, surface)	IgG	556341
Anti-LAMP1	Rabbit	Abcam	1:110 (ICC)	IgG	ab24170
Anti-myc	Mouse	Cell Signalling Technology	1:1000 (WB)	IgG	2276S
Anti-phosphoY421-cortactin	Rabbit	Invitrogen	1:500 (WB)	IgG	44-854G
Anti-phosphoY466-cortactin	Rabbit	Millipore	1:500 (WB)	IgG	AB3795
Anti-β-tubulin	Mouse	Sigma-Aldrich	1:10 000 (WB)	IgG	T8328

The secondary antibodies used are given in table 4-2.

Table 4-2 Secondary antibodies used for WB and ICC

Antibody	Supplier	Dilution
AlexaFluor488 anti-mouse	Life Technologies	1:500 (ICC)
AlexaFluor488 anti-rabbit	Life Technologies	1:500 (ICC)
AlexaFluor647 anti-mouse	Life Technologies	1:500 (ICC)
Dylight 405	Fisher Scientific	1:500 (ICC)
Anti-mouse HRP	GE Healthcare	1:10 000 (WB)
Anti-rabbit HRP	GE Healthcare	1:10 000 (WB)

4.1.2 Chemicals and solvents

All chemicals, acids and solvents were from Sigma-Aldrich unless otherwise stated. All additional manufacturers for materials are noted appropriately.

4.1.3 Buffers

The buffers used are given in table 4-3.

Table 4-3 Buffers

Buffer	Solutes	Solvent
Running Buffer	25mM tris(hydroxymethyl)-amino-methane (TRIS) 250mM glycine (Severn Biotech Ltd.) 0.1% SDS	ddH_2O
Transfer Buffer	24mM TRIS 192mM glycine 10% methanol	ddH_2O
Sample buffer (4x)	24mM TRIS pH 6.8	ddH_2O

	10% SDS 50% glycerol (Millipore) 0.01% bromophenol blue 0.05% β-mecaptoethanol (ThermoFisher)	
TRIS buffered saline with Tween (TBS-T)	137 mM NaCl 2.7 mM KCL 19 mM TRIS 0.1% Tween-20	ddH_2O
HEPES buffered saline (HBS)	25mM HEPES pH 7.4 140 mM NaCl 5 mM KCl 1.8 mM $CaCl_2$ 0.8 mM $MgCl_2$ 10 mM Glucose	ddH_2O
Borate Buffer	50mM boric acid 10mM sodium tetraborate	Cell Culture Sterile Water (GE Healthcare)
Milk	5% non-fat skimmed milk powder (Co-operative)	TBS-T
Bovine Serum Albumin (BSA)	5% BSA powder	TBS-T
TAE (TRIS-acetate-EDTA)	40mM TRIS-acetate 1mM ethylenediamine tetra acetic acid (EDTA)	ddH_2O
Homogenisation Buffer	50mM Tris pH7.4 150mM NaCl 0.1% SDS 1% Triton Pierce protease inhibitor Phosphatase cocktail I, II (Sigma-Aldrich)	ddH_2O

4.1.4 Bacterial reagents

4.1.4.1 Plasmids

All constructs utilised were acquired and/or cloned as outlined in table 4-4.

Table 4-4 Details of plasmid constructs used

Complementary DNA	Backbone	Tag/Reporter	Cloning sites
APP_{WT}	pcDNA3.1	pIRES-mCherry	3' SacII 5' NheI
APP_{MV}	pcDNA3.1	pIRES-mCherry	3' SacII 5' NheI
APP_{WT} (Kevin Wilkinson)	pcDNA3.1	pIRES-EGFP	3' SacII 5' NheI
APP_{MV} (Kevin Wilkinson)	pcDNA3.1	pIRES-EGFP	3' SacII 5' NheI
shRes-APP_{WT}	pXLG3	myc	3' MscI 5' SpeI
shRes-APP_{SWE} (Kevin Wilkinson)	pXLG3	myc	3' MscI 5' SpeI
sh-APP (Kevin Wilkinson)	pXLG3	EGFP	3' MscI 5' SpeI
sh-Scr (Kevin Wilkinson)	pXLG3	EGFP	3' MscI 5' SpeI

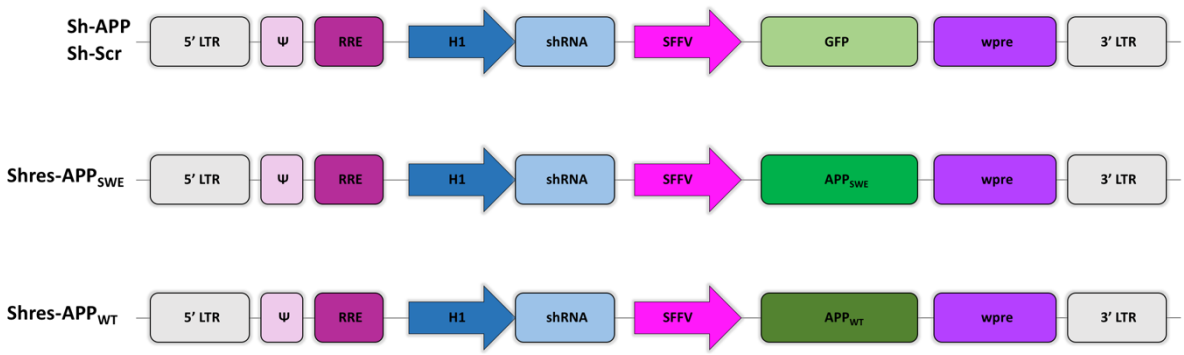

Figure 4-1 Schematic representation of molecular replacement constructs used.

Top panel shows knockdown and scrambled control constructs, middle panel shows APP_{SWE} knockdown replacement construct and bottom panel shows APP_{WT} knockdown replacement construct. Expression constructs of GFP, APP_{SWE} and APP_{WT} were made by cloning these genes into pXLG3 under a spleen-focus-forming virus (SFFV) promoter. Short hairpin RNAs (shRNAs) targeting APP or scrambled control were inserted into the pXLG3 plasmid under a H1 promoter. The Rev responsive element (RRE) and Ψ element provide regulation of virus production. The long terminal repeat (LTR) 5' serves as the promoter for the entire viral genome, while the LTR at the 3' provides the nascent viral RNA polyadenylation.

4.1.4.2 Escherichia coli (E.Coli)

Table 4-5 E.Coli strains and genotypes

Strain	Genotype
DH5α	*sup*E44 Δ*lac u*169 (Φ80 *lacZ*Δ M15) *hsd*R17 *rec*A1 *en*dA1 *gyr*A96 *thi*-1 *rel*A1
XL1-Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB laclqZΔM15 Tn10 (Tetr)]

4.1.4.3 Media

Luria-Bertani (LB) broth (ThermoFisher) was used to propagate plasmids. The appropriate antibiotic was added: ampicillin (100 µg/ml), kanamycin (25 µg/ml). 1.5% agar plates were made in-house in LB broth and supplemented with the appropriate antibiotic.

4.1.5 Cell culture reagents

4.1.5.1 Cell lines

Human embryonic kidney (HEK-293T) cells were obtained from The European Collection of Cell cultures, aliquoted and stored in 10% dimethyl-sulfoxide in a liquid nitrogen cryo-store.

4.1.5.2 Media and reagents

The cell culture media and reagents used are given in table 4-6. All are from Gibco unless otherwise stated.

Table 4-6 Cell culture media and reagents

Media	Components	Base Media
Neuronal Plating	1% GlutaMAX™ 1% Penicillin/Streptavidin (Sigma-Aldrich) 2% B-27 5% horse serum (Sigma-Aldrich)	Neurobasal
Neuronal Feeding	0.4% GlutaMAX™ 0.4% Penicillin/Streptavidin 2% B-27	Neurobasal
DMEM Complete	1% Penicillin/Streptavidin 10% fetal bovine serum (FBS) (Sigma-Aldrich)	DMEM (Sigma-Aldrich)

4.1.6 Plastic and glassware

Serological pipette tips (5-25ml) were from SARSTEDT, plastic Gilson pipette tips (10-1000µl) were from Starlab and gel loading tips from Fisher Scientific. Microcentrifuge tubes were from Eppendorf and PCR tubes from Starlab. 15ml and 50ml plastic conical tubes, universal and bijou tubes, and all cell culture dishes and plates were from Greiner Bio-One. Spectophotometer cuvettes were from Fisher Scientific and microscope slides were from BDH and glass coverslips from VWR international.

4.2 MOLECULAR BIOLOGY METHODS

To minimise cross-contamination, all microbial work was carried out using sterile media and equipment and all manipulations were carried out in close proximity to a Bunsen flame.

4.2.1 Transformation of E.Coli

DH5α or *XL1*-Blue cells were stored at -80°C and thawed on ice. Approximately 10ng DNA was added to 10-30µl competent cells and kept on ice for 30 minutes. Cells were heat shocked at 42°C for 90 seconds and then immediately chilled on ice for 2 minutes. 100µl LB broth was added to cells and incubated with shaking at 37°C for 60 minutes to allow for expression of antibiotic resistance. Cells were plated onto agar plates containing the appropriate antibiotic and placed into an incubator at 37°C for approximately 12-16 hours.

4.2.2 Colony amplification

Transformed colonies were picked and cultured in 100ml LB broth with the appropriate antibiotic. Cultures were grown whilst shaking at 37°C for 12-16 hours.

4.2.3 Preparation of DNA

Cultures were pelleted at 4000 xg for 10 minutes. GeneJET mini- and midi-preps (ThermoFisher) were used to extract and purify DNA according to the manufacturer's instructions. The concentration of purified DNA was measured using a NanoDrop ND-1000 (LabTech) at an absorbance wavelength of 260nm. Mini-preps ascertained the success of cloning, midi-preps amplified DNA quantities for mammalian cell transfection.

4.2.4 Polymerase chain reaction (PCR)

PCR reactions were prepared on ice using the high fidelity KOD hot start DNA polymerase kit (Millipore). Reactions were made up with 1x KOD polymerase buffer, 1.5mM $MgSO_4$, 0.2mM 2'-deoxynucleoside-5'-triphosphate (dNTPs), 1 Unit (U) KOD polymerase, 0.3µM forward and reverse primer, 10-100ng template DNA and made up to 50µl with deionised distilled water (ddH_2O). Reactions were placed in a thermal cycler and the PCR programme set up as in table 4-7. The

denaturing-annealing-extension cycle (highlighted in green) was repeated 25 times. PCR products were purified using the GeneJET Gel Extraction Kit (ThermoFisher) according to the manufacturer's instructions.

Table 4-7 PCR cycling programme

	Target Size			
Step	**<500bp**	**500-1000bp**	**1000-3000bp**	**>3000bp**
Polymerase activation	95°C for 2 minutes			
Denaturing	95°C for 20 seconds			
Annealing	55°C for 10 seconds			
Extension	70°C for 10s/kb	70°C for 15s/kb	70°C for 20s/kb	70°C for 25s/kb
Final extension	70°C for 4 minutes			
Cooling	15°C for 10 minutes			

4.3 CONSTRUCT CLONING

4.3.1 PCR product purification

Agarose gels (0.8% or 1.5%) were used to resolve digested PCR products and vectors for screening of positive clones. Molecular biology grade agarose (Sigma) was dissolved in 0.5x TAE buffer whilst heating. Once cooled to approximately 50°C, ethidium bromide was added at a final concentration of 0.5µg/ml and the solution was poured into a gel mould with a comb to set. DNA samples were mixed with 6x loading dye (New England Biolabs) to a final concentration of 1x and 5µl loaded into wells alongside 3µl Hyperladder (Bioline). Gels were run using Mupid-eXu gel apparatus for 30 minutes at a constant 135 V in 0.5x TAE buffer and bands detected on an ultra-violet (UV) transilluminator.

4.3.2 Restriction digests

Restriction digests were set up with 1x NEB buffer 1-4 depending on enzyme activity compatibility in 100µl total volume. 1-5µg plasmid DNA or PCR product was incubated with 20U of each restriction enzyme at 37°C for 2 hours. 20U Calf intestinal alkaline phosphatase (CIP) was added to host plasmid digestion mixtures to catalyse the removal of 5' phosphate groups from the host DNA, thus preventing re-ligation. Products were purified and digestion validated by running on agarose gel.

4.3.3 Ligation

5µl of purified DNA was run on agarose gel to determine a suitable vector:insert ratio for ligation. Typically, a 1:3 molar ratio was used to mix vector and insert. 1 volume of insert and vector was added to 1 volume Takara T4 ligase solution 1 (Biowhittaker) and ligation was left for 1-2 hours at room temperature. A control was set up alongside where insert was replaced with ddH_2O to determine the level of spontaneous re-ligation. Ligation reactions were then transformed as described in 3.2.1.

4.3.4 Confirmation of cloning

Small scale PCR reactions and restriction digests were used for screening of successful clones. DNA sequencing was performed by Eurofins Genomics using stock vector primers or specifically designed primers corresponding to the expected sequence of the insert.

4.4 HEK293T CELL CULTURE

4.4.1 Routine culture conditions

All cell culture methods were carried out in laminar fume hoods with aseptic technique. HEK293T cells were cultured in complete Dulbecco's Modified Eagle Medium (DMEM). Cells were cultured in humidified 37°C incubators with 5% CO_2.

4.4.2 Cell passage

Cells were routinely passaged once 70-90% confluent. Media was aspirated and cells were washed in 8ml pre-warmed 1x distilled phosphate buffered saline (DPBS, Sigma) in Cell Culture Sterile Water (GE Healthcare). Cells were then incubated with 1ml of 0.025% trypsin-EDTA for 2 minutes at room temperate to detach adherent cells. Cells were diluted in 10ml complete media, pelleted by centrifugation at 2000 xg for 2.5 minutes and re-suspended in 10ml complete media by trituration. 1ml cell suspension was transferred into a new T75 flask containing 20ml complete media.

4.4.3 Preparation of lentivirus

4.4.3.1 HEK293T cell preparation

Following passage, HEK cells were counted at a 1:10 dilution in 0.4% trypan blue solution using a haemocytometer. 1.8 million cells were plated per 60mm cell culture dish containing 3ml complete media and incubated overnight at 37°C or until 70% competency was reached.

4.4.3.2 HEK293T cell transfection

A three-plasmid 2nd generation lentiviral-producing system was used through transfection of HEK293T cells. DNA-DMEM was prepared using 1ml sterile-filtered (45µm nylon mesh, Fisher Scientific) plain DMEM, 4µg XLG viral vector, 1µg pMD2.G (packaging vector) and 3µg p8.91 (helper vector), per virus to be made. Polyethylenimide (PEI)-DMEM was prepared with 1ml sterile-filtered plain DMEM per transfection, plus 1ml extra, combined with 24µl of 1mg/ml PEI for each ml of DMEM. This was mixed by inverting and left to equilibrate at room temperature for 2-3 minutes. 1ml of the PEI-DMEM mixture was then added to each DNA-DMEM mixture, inverted several times and left at room temperature for 30 minutes.

HEK293T cells were washed once with 3ml pre-warmed plain sterile-filtered DMEM, then media aspirated and 2ml of the transfection mix added. Cells were incubated for 4 hours at 37°C before replacing media with 3ml pre-warmed sterile-filtered complete DMEM.

4.4.3.3 Harvesting lentivirus

38-48 hours post-transfection the virus-containing media was centrifuged at 4000 xg for 10 minutes to pellet any remaining cell debris. The resulting supernatant was then filtered using a 0.45µm membrane pre-wetted with neuronal plating media. Viruses were aliquoted and stored at -80°C until use.

4.5 ANIMAL HUSBANDRY

All animal experimental procedures were performed in accordance with the "Animals Scientific Procedures Act 1986" and the University of Bristol and University of Exeter policy on working with animals.

Wistar rats were bred at the University of Bristol animal facilities and kept in individually ventilated cages with food and drink available ad libitum.

J20 and wild-type mice were bred at the University of Exeter animal facilities and kept in individually ventilated cages under a 12 h:12 h light:dark schedule with food and drink available ad libitum.

4.6 DISSOCIATED RAT EMBRYONIC NEURONAL CULTURE

4.6.1 Glass coverslip preparation

22mm borosilicate glass coverslips were washed in 100% nitric acid overnight with gentle shaking. Coverslips were then washed three times in cell culture grade water, three times for 30 minutes in cell culture grade water and then with 70% ethanol for 2 hours. Another three 30 minute washes in cell culture grade water followed before a final three quick washes. Each coverslip was placed into a 35 mm neuronal cell culture dish and coated with 1mg/ml Poly-D-lysine (PDL, Sigma) diluted in borate buffer for 6-8 hours at 37°C. Following three washes in cell culture grade water and one wash in plating media, on the day of dissection plating media was replenished and returned to 37°C until cell seeding.

4.6.2 Dissection

Primary dissociated hippocampal and cortical cultures were prepared from embryonic day 18 (E18) Wistar rat brains. All dissections were carried out according to a modified version of the Brewer method (Brewer 1997) in hank's balanced salt solution (HBSS, Gibco) at room temperature. Pregnant mothers were sacrificed by cervical dislocation under Schedule 1 of the Home Office Animal Welfare Regulations. The embryonic sac was removed and placed into HBSS. Embryos were removed from the embryonic sac and decapitated using standard dissection tools. Under a dissection microscope, meninges were removed and cortices and hippocampi excised. These were then washed three times in 10ml HBSS and incubated in a water bath at 37°C with 1ml 0.025% trypsin-EDTA (pH 7.2) in 10ml HBSS for 9 minutes or 3ml 0.025% trypsin-EDTA in 30ml HBSS for 12 minutes for hippocampi and cortices respectively. Following trypsinisation, hippocampi and cortices were given another 3 washes in HBSS and a wash in plating media. Hippocampi and cortices were triturated in 1ml and 5ml of plating media and cell suspensions were then diluted to total volumes of 5ml and 20ml respectively. The cortical cell suspension was then filtered (70µm nylon mesh, Fisher Scientific) to remove non-dissociated tissue. Cells were counted in a 1:10 dilution in 0.4% trypan blue solution using a haemocytometer.

4.6.3 Routine culture conditions

Hippocampal neurons were plated at 200,000 cells per 22mm borosilicate glass coverslip and cortical neurons were plated at 500,000 cells per PDL-coated 35mm neuronal cell culture dish. Cells were incubated at 37°C with 5% CO_2. 2-16 hours after plating, media was replaced with feeding media and one week after plating 1ml feeding media treated with fluorodeoxyuridine and uridine was added to inhibit glial growth. Cells were then incubated until required.

4.6.4 Neuronal transfection

For each coverslip 1-2µg DNA and 2-3µl Lipofectamine 2000 (L2K, Invitrogen) were separately diluted in pre-warmed neurobasal medium and left to equilibrate for 5 minutes. The L2K was then added to the DNA at a ratio of 1.5µl:1µg, vortexed and incubated for 20 minutes at room temperature. At 13 days in vitro (DIV13) hippocampal neurons were washed once in pre-warmed

plain neurobasal and then placed into pre-warmed plain neurobasal. 200µl of DNA-L2K complex was pipetted dropwise onto each coverslip and incubated at 37°C for 45 minutes. Neurons were then returned to their original media via another wash in pre-warmed plain neurobasal and used for experiments 24-48 hours later. Transfection efficiency was typically around 10%.

4.6.5 Neuronal viral infection

Virus' were thawed and cortical neurons infected at DIV12-16. For each 35mm neuronal cell culture dish, a maximum of 450µl lentivirus was added. Neurons were then left at 37°C for 4 days until lysis.

4.7 WESTERN BLOTTING

4.7.1 Sample preparation

Cortical neurons were lysed in 250µl 1x sample buffer and, using a cell scraper, were removed from the dish surface and transferred into Eppendorf tubes. Samples were then boiled for 10 minutes at 95°C, centrifuged briefly at 13,000 xg and stored at -20°C.

4.7.2 SDS-PAGE

Protein separation was performed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using Bio-Rad Mini-Protean II equipment. 8-10% acrylamide resolving gels (8-10% (w/v) acrylamide, 375MM Tris-HCl pH8.8, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate (APS), 0.01% (v/v) Tetramethylethylenediamine (TEMED)) were polymerised at room temperature in 1.5mm glass plates (Bio-Rad). Different percentages of acrylamide were used in resolving gels according to the molecular weight of the protein being analysed. 8% gels and 10% gels resolved proteins of molecular weight 40-250 kDa and 30-200 kDa respectively. 5% stacking gel (5% (w/v) acrylamide, 125mM Tris-HCl pH6.8, 0.1% (w/v) SDS, 0.1% (w/v) APS, 0.01% (v/v) TEMED) were polymerised on top of the resolving gel and set with a 10 or 15 well gel comb to create sample wells.

Polymerised gels were mounted into electrophoresis tanks (Biorad) and immersed in 1x SDS-PAGE running buffer (pH 8.3). Samples were loaded into wells alongside 3µl molecular weight marker

(Page Ruler™ Prestained Protein Ladder) and run at 100 volts through the stack and 150-180 volts thereafter.

4.7.3 Wet transfer

Following electrophoresis, resolved proteins were transferred onto Polyvinylidene Difluoride (PVDF) membrane. Membranes were activated for 30 seconds in 100% methanol and then stored in transfer buffer (pH 8.3). 3MM Whatman blotting paper and sponges were also placed in transfer buffer. The gel was excised from the glass plates and stacking gel removed before assembly of the transfer in apparatus according to manufacturer's instructions (Bio-Rad). The transfer system was submerged in 1x transfer buffer and run for 60 minutes at 400mA with buffer agitation and cooling.

4.7.4 Immunoblotting

Following transfer onto PVDF membranes, proteins were detected via immunoblotting. Membranes were removed from the transfer apparatus and placed in blocking buffer (pH 7.4) (5% (w/v) milk or 5% (w/v) BSA if phospho-proteins were being detected) for 1 hour on a shaker at room temperature. Blocking buffer was then taken off the membrane and replaced with primary antibody diluted in blocking buffer (table 4-1) overnight at 4°C with shaking. Membranes were washed 3 times for 5 minutes in 1x TBS-T before incubation with the appropriate horseradish peroxidase (HRP) conjugated secondary antibody (table 4-2) for 1 hour at room temperature. Membranes were then washed 4 times for 10 minutes in 1x TBS-T before being developed.

4.7.5 Chemodetection of PVDF membranes

In order of increasing strength, the following ehanced chemoluminescence (ECL) reagents were utilised depending on the concentration of protein present in the membrane: Luminata Classico Western horseradish peroxidase (HRP) substrate (Millipore), Luminata Crescendo Western HRP substrate (Millipore), SuperSignal™ West Femto Maximum Sensitivity Substrate (Fisher Scientific). Membranes were incubated with 1ml of reagent for 1 minute at room temperature, before being transferred to saran wrap in a developing cassette (Amersham) which was sealed to avoid bubbles.

Proteins were visualised by exposing membranes to radiographic film (Kodak) for 15 seconds to 5 minutes, and then processing in a mini developer (Konica SRX-101A medical film processor).

4.7.6 Stripping and re-probing of PVDF membranes

Membranes were incubated with Restore™ PLUS Western Blot Stripping Buffer (Fisher Scientific) according to manufacturer's instructions, allowing the membrane to be re-probed with a different antibody as described.

4.7.7 Quantification of immunoblots

Films were scanned at 600dpi and saved as TIFF files. The images were analysed in ImageJ software (NIH) by scanning densitometry. Bands were manually outlined using the rectangular shape selection tool and the pixel intensity measured as the area under the peak.

Data was processed in Microsoft Excel and normalised as appropriate. Values were exported to GraphPad Prism for statistical analysis.

4.8 PROTEIN BIOCHEMISTRY METHODS

4.8.1 J20 mice

At 16-18 months of age, J20 mice were sacrificed under Schedule 1 of the Home Office Animal Welfare Regulations. Cortices, hippocampi and cerebella were dissected and snap-frozen on dry ice. They were then stored at -80°C until homogenised.

4.8.2 Brain homogenisation

All utensils and a pestle and mortar were pre-chilled on dry ice. Frozen brain tissue was cut into small pieces with a scalpel, transferred into a pestle and mortar and covered in liquid nitrogen. The liquid nitrogen was left to evaporate and then the tissue ground into a fine powder. A chilled spatula was used to transfer the powder into an eppendorf, and the eppendorf stored in an urn of liquid nitrogen. This was repeated for all samples. 250µl of chilled homogenisation buffer was then added to each sample before placing on ice. Tissue was sonicated on ice and then centrifuged for 20 minutes at 16,000 xg and 4°C. A Bradford assay was carried out on the supernatant to determine protein concentrations.

4.8.3 Bradford assay

Protein concentration was determined following a modified version of the Bradford method (Bradford, 1976) and using BioRad reagent. BSA standard curves were created with serial dilutions from 0.1mg/ml to 2mg/ml. BioRad reagent was diluted 1 in 5 with ddH_20 and 995µl was added to 5µl of each sample in a 1ml cuvette. A spectrophotometer was normalised to the homogenisation buffer and optical density measured at a wavelength of 595nm. A total of 3 readings were taken per sample, averaged and then plotted against the BSA standard curve. Protein concentrations were extrapolated accordingly.

4.9 IMMUNOCYTOCHEMISTRY

4.9.1 Total immunocytochemistry

For total staining, DIV15 hippocampal neurons were fixed in 4% paraformaldehyde (PFA)/4% sucrose/1x Dulbecco's (D)PBS for 15 minutes, washed 3 times in 1xDPBS, quenched in 0.1M glycine/1xDPBS for 5 minutes and then washed another 3 times in 1xDPBS. Permeabilisation in 0.1% Triton X-100/5%BSA/1xDPBS for 10 minutes followed. Coverslips were then incubated in primary antibody diluted in 5%BSA/1xDPBS for 1 hour, washed 3 times in 1xDBPS and incubated with AlexaFluor488 secondary antibody diluted in 5%BSA/1xDPBS for 45 minutes. Coverslips were

washed 5 times in 1xDPBS before being mounted onto glass slides using Fluoromount G (Fisher Scientific).

4.9.2 Antibody feeding assay

For live surface staining, coverslips were incubated with HBS pre-warmed to 37°C for 6 minutes before being incubated in primary antibody diluted in HBS for 20 minutes. Coverslips were washed in HBS and returned to conditioned media for either 20 or 45 minutes at 37°C (figure 4-2). Neurons were fixed with 4%PFA/4%sucrose/1xDPBS for 5 minutes, washed 3 times in 1xDPBS, quenched in 0.1M glycine/1xDPBS and washed another 3 times in 1xDPBS. Coverslips were then incubated with AlexaFluor488 secondary antibody diluted in 5%BSA/1xDPBS, washed 5 times in 1xDPBS and fixed again in 4%PFA/4%sucrose/1xDPBS for 12 minutes. Neurons were permeabilised for 10 minutes in 0.1% Triton X-100/5%BSA/1xDPBS and then incubated with lysosomal-associated membrane protein 1 (LAMP1) primary antibody diluted in 5%BSA/1xDPBS for 1 hour. 3 washes in 1xDPBS followed and incubation with AlexaFluor647 and Dylight405 for 45 minutes. Coverslips were then washed and mounted as described in section 4.8.1.

In order to block lysosomal degradation, 100 µg/ml leupeptin was added to the culture medium for 3 hours before and during primary antibody incubation and trafficking.

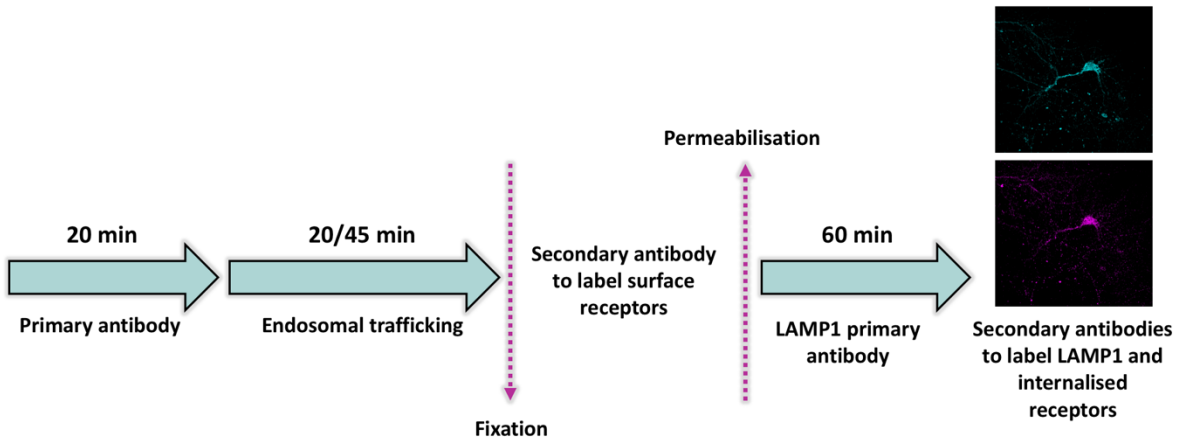

Figure 4-2 Timeline of major steps in antibody feeding immunocytochemistry protocol.

4.9.3 Image acquisition and analysis

Images were acquired on an SP5-AOBS (Leica) confocal laser scanning microscope attached to a DM I6000 (Leica) inverted epifluorescence microscope using a 40x or 63x oil immersion objective lens. For total immunocytochemistry, the Leica application suite software was used to acquire 0.5-µm stepped Z-stacks throughout the cell depth and maximum projections were taken for each series. Images were obtained with a resolution of 1024x1024 pixels at 1x optical zoom, a pinhole of 1 airy unit (AU) at 400Hz averaged 6 times. Acquisition parameters were kept constant across all coverslips per experiment. Images were analysed using ImageJ software.

4.10 STATISTICAL ANALYSIS

Data are presented as mean ± standard error of the mean (SEM). Mean gray values normalised to the MV control conditions separately for each dissection which accounted for differences between experiments carried out on neuronal cultures from different dissections.

For total immunocytochemistry, the n value given for each experiment refers to the number of coverslips (taken from different dissections) and each data point is an average of 3 cells. Average mean gray value was measured for each cell and processed for statistical evaluation.

For antibody feeding assays, three 30-40µM portions of distal dendrites were analysed per cell. The n value refers to the number of coverslips (taken from different dissections) with each coverslip averaged from three cells with three ~30-40µm portions of dendrite per cell. Pearson's co-localisation analysis was performed using the Coloc 2 plugin in ImageJ.

For western blotting experiments on J20 mice, the n value refers to the number of brain homogenisation preparations, with each preparation containing five WT and six J20 mice. For western blotting experiments on cortical neuronal cultures, the n values refer to the number of cortical cultures.

Data were first tested for normality using the Shapiro-Wilk normality test to determine the appropriate statistical test. If found to fit a normal distribution, data were analysed using parametric statistics. Two-tailed Student's t-tests were used for comparing 2 groups of data and a one-way analysis of variance (ANOVA) used when 3 or more groups were compared. A Bonferroni's post-hoc test for multiple comparisons was used to compare all variables with one another.

Data sets exhibiting non-normal distributions were analysed with non-parametric statistical tests. Experiments with 2 variables were analysed with Mann Whitney tests for unpaired data and Wilcoxon matched-pairs signed rank tests for paired data. Experiments with more than 2 variables were analysed with Kruskal-Wallis one-way ANOVAs. All statistical tests were performed on GraphPad Prism (v8.0). Differences were considered significant if * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

5 GluA1 and GluA2 degradation in Alzheimer's disease models

5.1 Aims

The first aims of my project were to investigate the total expression levels of the AMPAR subunits GluA1 and GluA2, which can indicate whether a change in subunit degradation is witnessed in the disease state *in vitro*. Subunit expression was examined in three separate models of AD to determine similarities and distinctions that might arise between different models mimicking distinct pathological characteristics of the disease. It was hypothesised that expression levels of GluA1 and GluA2 would be reduced in all three models due to their degradation in response to amyloidopathy.

5.2 Introduction

Although both tau and Aβ are key to the pathogenesis of AD, strong evidence from genetics and transgenic mice has implicated Aβ to be strongly associated with disruption of synaptic function (Guntupalli, Widagdo and Anggono, 2016). Furthermore, the genes firmly indicated in the pathophysiology of familial AD all result in Aβ pathogenesis, allowing this hallmark of the disease to be modelled (Reitz, Brayne and Mayeux, 2011). Thus, the most commonly used experimental models of AD *in vivo* are transgenic mice that overexpress human genes associated with familial AD and mimic the formation of Aβ plaques (Drummond and Wisniewski, 2017). It was discovered that mouse strains expressing numerous familial AD mutations at once exhibit more severe pathology (Mucke *et al*., 2000). As a result, models such as the J20 mouse strain were developed, which expresses both the Swedish ($APP_{K670N/M671L}$) and Indiana (APP_{V717F}) mutations (Mucke *et al*., 2000) and exhibits a host of AD phenotypes comprising deficits in both LTP (Saganich *et al*., 2006) and cognition (Cheng *et al*., 2007; Wright *et al*., 2013).

Modelling amyloidopathy *in vitro* is often afforded by neuronal cell cultures which provide an easily manipulated and controlled environment for revealing molecular and cellular mechanisms of disease states (Akhter, Sanphui and Biswas, 2014). There are a number of techniques that can be implemented to replicate the actions of Aβ in culture, including APP overexpression. This approach has previously been validated and demonstrated to result in AD pathology such as a loss of spine and dendritic AMPAR subunits (Kamenetz *et al*., 2003; Hsieh *et al*., 2006). A better technique to mimic

the pathological hallmarks of amyloidopathy in culture would be the endogenous expression of APP with human mutations. This would be advantageous because not only would it allow APP with clinically relevant familial mutations to be expressed, but they would be expressed at endogenous levels which avoids any artefacts that might result from protein overexpression (Kuang *et al*., 2006).

Since AMPARs mediate the overwhelming majority of fast excitatory transmission in the central nervous system, changes to their composition and properties at the post-synaptic membrane significantly modify synaptic function and neuronal connectivity. Indeed, the synaptic dysfunction that is a characteristic of AD can be explained by a loss of the AMPAR subunits GluA1 and GluA2 from the synaptic surface (Hsieh *et al*., 2006; Liu *et al*., 2010; Zhao *et al*., 2010; Miñano-Molina *et al*., 2011; Alfonso *et al*., 2014). Aβ facilitates the loss of surface AMPAR levels via a range of mechanisms including: the activation of calcineurin and subsequent dephosphorylation of AMPARs at Ser-845 (Zhao *et al*., 2009; Miñano-Molina *et al*., 2011); the phosphorylation of AMPARs at Ser-880 and subsequent dissociation from GRIP (Liu *et al*., 2010); the increased association of AMPARs with PICK1 (Alfonso *et al*., 2014); altered localisation of CaMKII at the synapse (Gu, Liu and Yan, 2008); and reduced synaptic expression of PSD-95 (Gylys *et al*., 2004; Almeida *et al*., 2005; Proctor, Coulson and Dodd, 2010; Sultana, Banks and Butterfield; 2010). Although some of these mechanisms suggest that receptors are lost from the surface via an increase in AMPAR endocytosis, another mechanism that could be responsible for receptor loss from the surface is a disruption to their recycling. Indeed, instead of being trafficked to recycling endosomes (REs), receptors can be targeted to late endosomes (LEs) where they are subsequently degraded (van der Sluijs and Hoogenraad, 2011; Hu *et al*., 2015). If this were happening, a loss of total AMPAR expression would be expected.

The levels of GluA1 and GluA2 protein expression have been examined in AD brain tissue. Indeed, immunocytochemical and biochemical techniques reveal that both GluA1 (Armstrong *et al*., 1994; Yasuda *et al*., 1995) and GluA2 (Armstrong *et al*., 1994; Yasuda et al., 1995; Ikonomovic *et al*., 1997; Carter *et al*., 2004) protein levels are reduced, particularly in the entorhinal cortex and subiculum of AD post-mortem brains. Although not all of the literature demonstrates this reduction, this conflict occurs in studies where messenger (m)RNA rather than protein levels were being examined (Pellegrini-Giampietro, Bennett and Zukin, 1994). Since a change to protein expression can indicate transcriptional down-regulation of protein messenger (m)RNA or changes in protein degradation (Goo, Scudder and Patrick, 2015), this suggests that these subunits are being degraded rather than any change to transcriptional control. On balance the evidence suggests that these AMPAR subunits are vulnerable to down-regulation in the disease state.

Based on the observation that synapses get weaker in AD rodent models (Cullen *et al*., 1997; Walsh *et al*., 2002; Wang *et al*., 2002; Shanker *et al*., 2007, Shanker *et al*., 2008) AMPARs are lost from synapses in vitro (Hsieh *et al*., 2006; Liu *et al*., 2010; Zhao *et al*., 2010; Miñano-Molina *et al*., 2011; Alfonso *et al*., 2014), and AMPARs are, generally speaking, reduced in post-mortem AD brains compared to controls (Armstrong *et al*., 1994; Yasuda *et al*., 1995; Ikonomovic *et al*., 1997; Carter *et al*., 2004), it was hypothesised that AMPARs may be degraded in response to amyloidopathy and thus their expression would be reduced.

This chapter examined the expression of AMPAR subunits in three different models of amyloidopathy. Since GluA1 and GluA2 predominate in the mature hippocampus, it was decided to focus on the total expression of these subunits (Wenthold *et al*., 1996; Lu *et al*., 2009). The three models that were investigated each mimicked Aβ pathogenesis in different ways: the overexpression of APP_{WT} in culture, the expression of a familial APP mutation at endogenous levels in culture, and a transgenic mouse strain carrying two familial APP mutations.

5.3 RESULTS

5.3.1 Overexpression of APP_{WT} causes a loss of total GluA2

The first model of amyloidopathy used to look at GluA1 and GluA2 degradation was the transfection of hippocampal neuronal cultures with APP_{WT} or APP_{MV}. It has previously been demonstrated that the overexpression of APP_{WT} results in greater production of Aβ, leading to AD phenotypes including synaptic removal of AMPARs, dendritic spine loss and the occlusion of LTD (Kamenetz *et al*., 2003; Hsieh *et al*., 2006). APP_{MV} is a point mutant of APP with a M596V mutation (Citron, Teplow and Selkoe, 1995) and can be used as a suitable control construct since it can only be cleaved by α-secretase and not β-secretase (Kamenetz *et al*., 2003). Thus, differences between APP_{WT} and APP_{MV} expressing cells are caused by BACE activity and Aβ generation. Indeed, APP_{MV} had no effect on glutamatergic synaptic transmission, while APP_{WT} overexpression depressed transmission which was attributable to Aβ production (Kamenetz *et al*., 2003). Differences in GluA1 or GluA2 expression levels observed between the effects of overexpressing APP_{WT} or APP_{MV} should therefore be attributable to increased levels of Aβ.

Changes to total AMPAR expression, as well as a change to transcriptional control, can indicate a change in degradation and can be visualised by labelling subunits under permeabilising conditions to measure both surface and internalised receptors. Experiments were carried out in DIV15 hippocampal neuronal cultures transfected with APP_{WT} or APP_{MV} for 48 hours. Immunocytochemistry was then carried out whereby neurons were fixed and permeabilised before total staining for GluA1 and GluA2 to investigate if Aβ production affected total GluA2 or GluA1 expression.

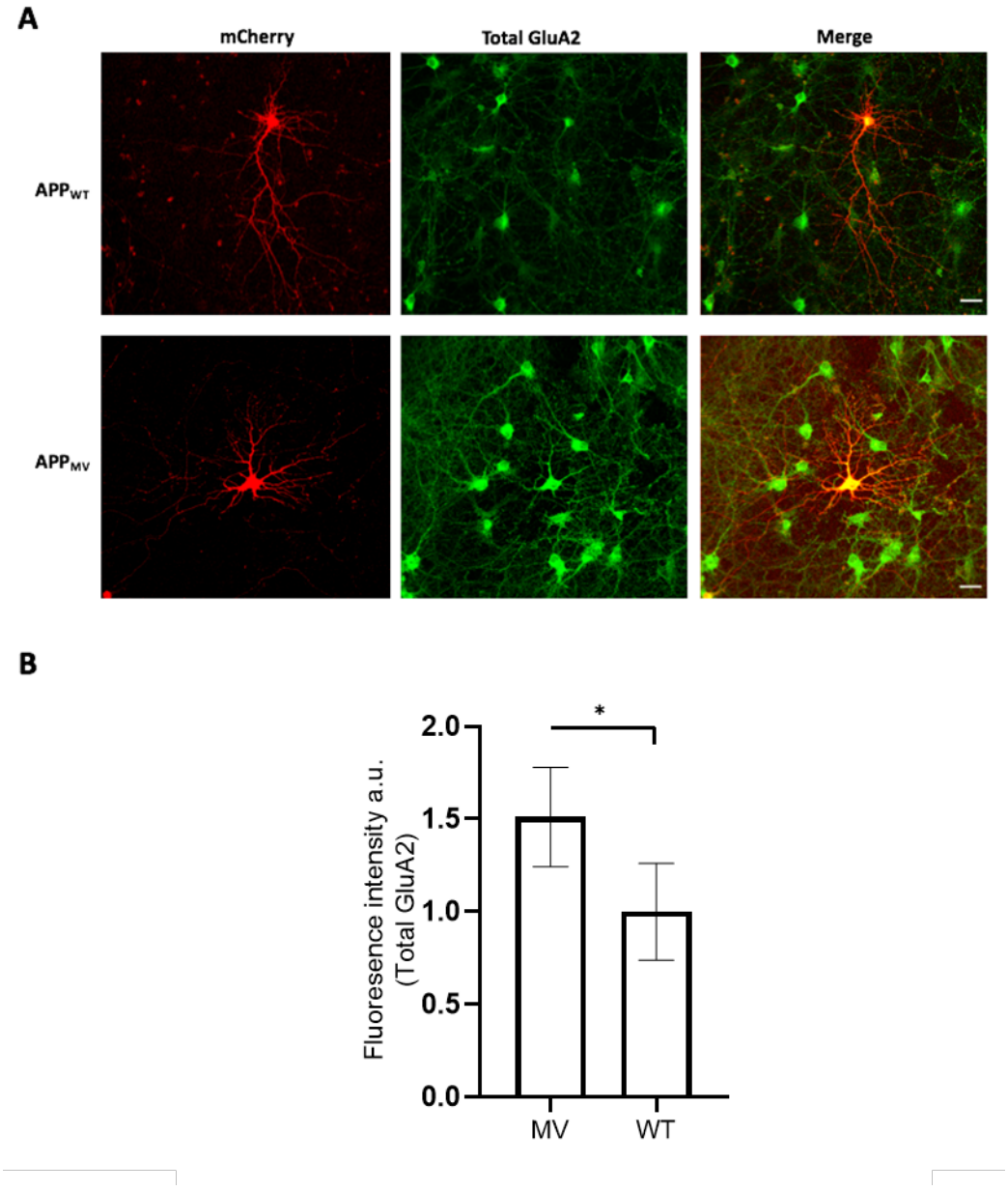

Figure 5-1 Overexpression of APP_{WT} in hippocampal neuronal cultures reduces total GluA2 expression.

(A) DIV13 primary hippocampal neurons were transfected with plasmids encoding APP_{WT}-IRES-mCherry or APP_{MV}-IRES-mCherry and returned to conditioned media for 48 hours. Cells were then fixed at DIV15, permeabilised and labelled for total GluA2. Transfected cells were identified by mCherry fluorescence (red channel). The green channel represents staining for total GluA2. Scale bar=30µm.

*(B) Quantification of mean fluorescence intensity of total GluA2 in transfected neurons. n=5. Paired two-tailed student's t-test, *p=0.0365.*

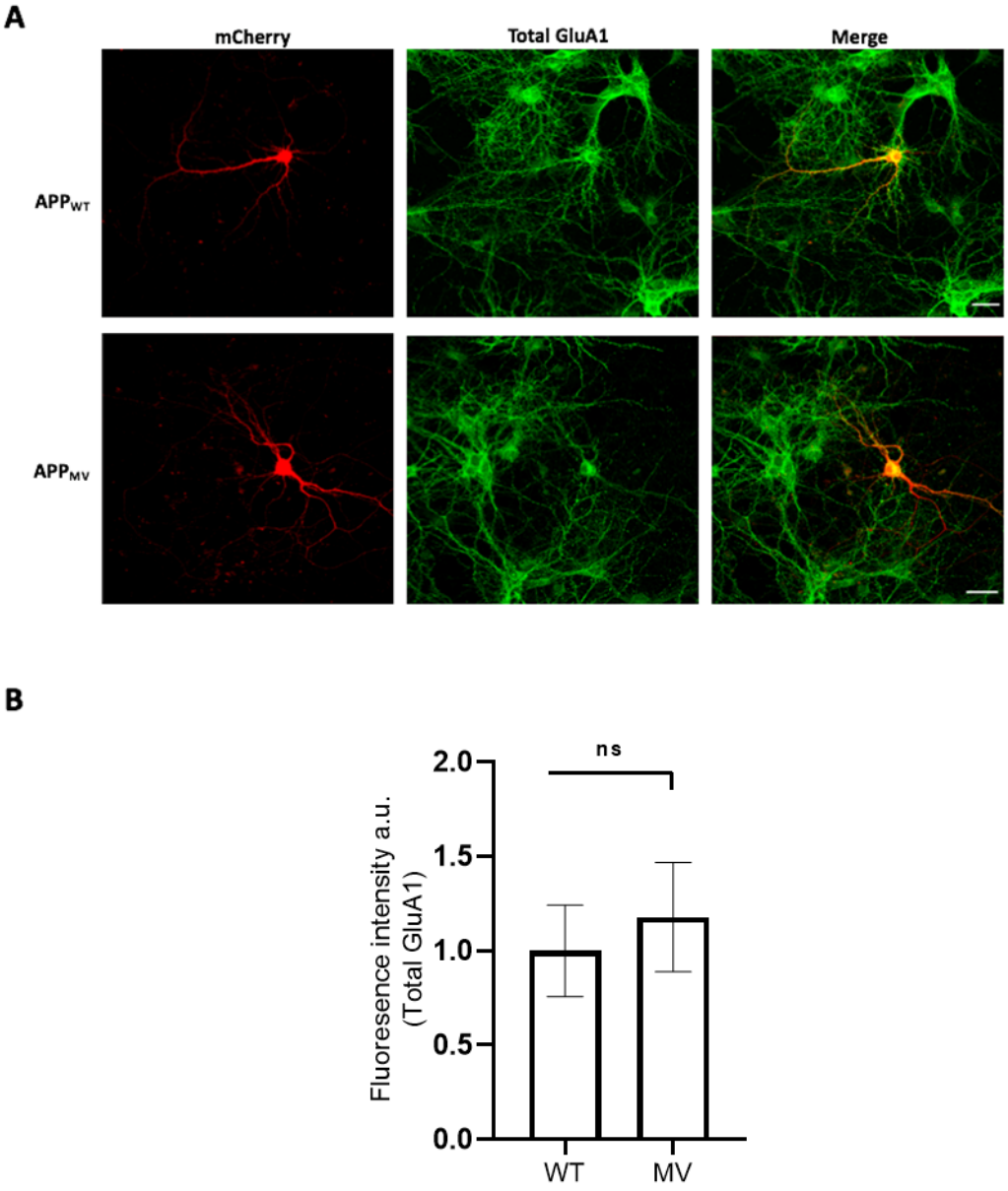

Figure 5-2 Overexpression of APP$_{WT}$ in hippocampal neuronal cultures does not alter total GluA1 expression.

(A) DIV13 primary hippocampal neurons were transfected with plasmids encoding APP$_{WT}$-IRES-mCherry or APP$_{MV}$-IRES-mCherry and returned to conditioned media for 48 hours. Cells were then fixed at DIV15, permeabilised and labelled for total GluA1. Transfected cells were identified by mCherry fluorescence (red channel). The green channel represents staining for total GluA1. Scale bar=30µm.

(B) Quantification of mean fluorescence intensity of total GluA1 in transfected neurons. n=5. Paired two-tailed student's t-test, p=0.2241.

Figure 5-1 showed that APP_{WT} overexpression decreased total GluA2 expression compared to APP_{MV} control cells. In contrast, figure 5-2 demonstrated that GluA1 expression levels were not affected by APP_{WT} overexpression, since there was no significant difference compared to APP_{MV} cells. Therefore, only total GluA2 levels were shown to decrease in response to APP_{WT} overexpression, indicating that elevated Aβ might be causing the selective entry of GluA2-containing AMPARs into a degradative path. Figure 5-1 A demonstrates that all neurons in the field of view transfected with APP_{WT} have reduced GluA2 expression levels compared to control neurons, which suggests Aβ secreted from the transfected neuron could be affecting expression in neighbouring cells.

5.3.2 Validation of APP molecular replacement lentiviral constructs

The second AD model used to look at GluA1 and GluA2 degradation was the infection of cortical neuronal cultures with APP_{SWE} and APP_{WT} knockdown-replacement viruses. This was a technique developed by our lab to replace endogenous APP with a clinically relevant familial mutation in culture. Whilst APP_{SWE} had already been developed, during the project we cloned an APP_{WT} control which allowed these viruses to be used for the first time to explore the molecular mechanisms of the disease. Viral delivery of shRNAs provides an efficient means to suppress endogenous or heterologous gene expression in cultured cells without the complications related to genetic ablation of a protein (Fellmann and Lowe, 2014). Furthermore, this technique was superior to the previous model because it allowed the expression of clinically relevant mutations in culture and avoided any artefacts that might result from protein overexpression.

It was first necessary to validate whether the molecular replacement constructs could knockdown endogenous APP and replace it with an appropriate level of APP_{WT} or APP_{SWE} in cortical neurons. Cortical neurons were used in this case instead of hippocampal due to time constraints so that a high density of neurons could be plated for Western blots. Cortical neuronal cultures were transduced with: the scrambled shRNA (Scr) with an enhanced green fluorescent protein (EGFP) reporter; APP shRNA (Sh-APP) with a GFP reporter; the APP_{WT} knockdown replacement construct (Shres-APP_{WT}) which expresses APP shRNA as well as human APP with a C-terminal myc tag; or the APP_{SWE} knockdown replacement construct (Shres-APP_{SWE}) which also expresses APP shRNA as well as human

APP with a C-terminal myc tag. Cells were then lysed 4 days later and samples immunoblotted for APP, myc and β-tubulin.

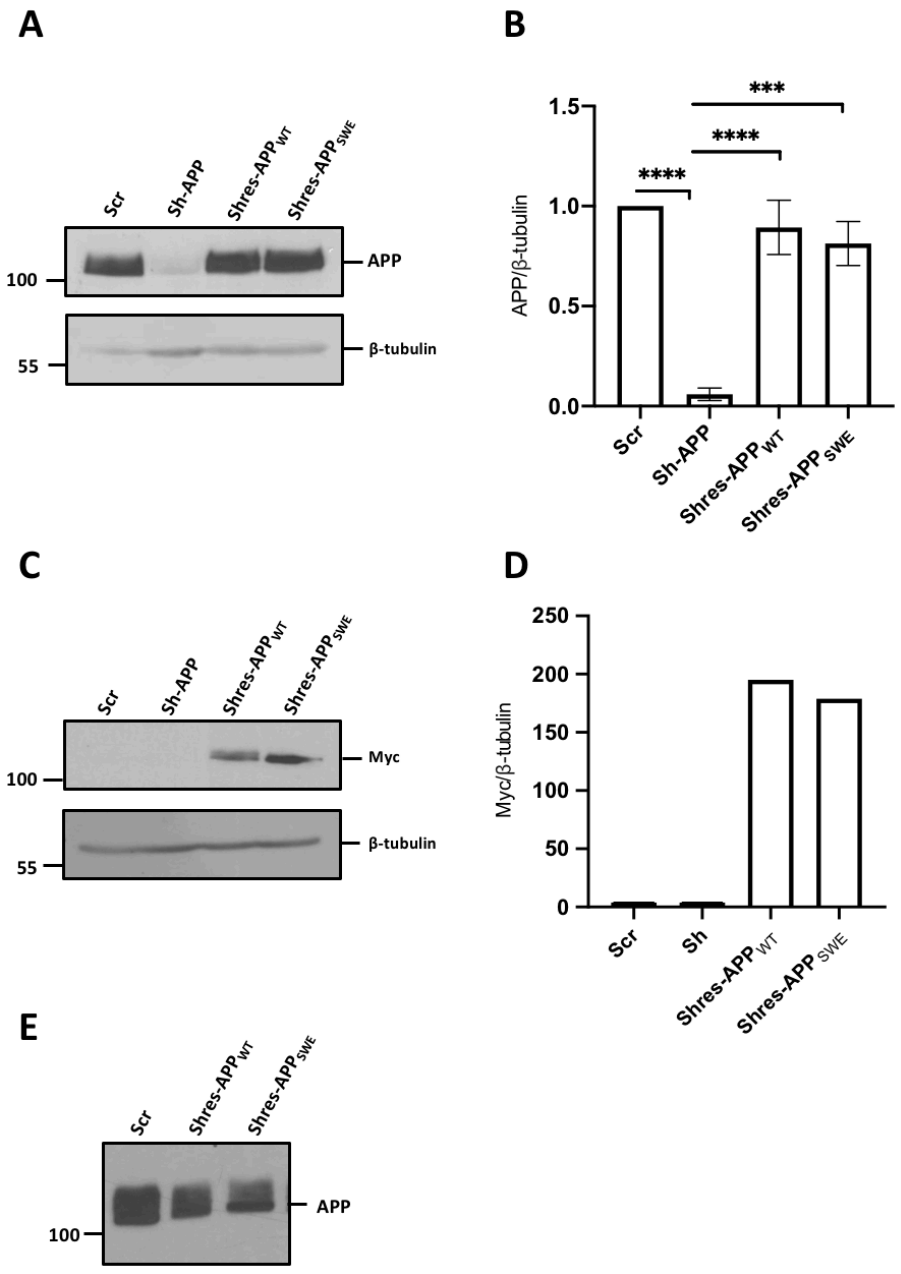

Figure 5-3 Validation of APP knockdown and replacement with myc-tagged APP_{WT} and APP_{SWE}.

(A) DIV12-16 cortical neurons were transduced with lentiviral molecular replacement constructs and returned to conditioned media for 4 days. Cells were then lysed and immunoblotted for APP and β-tubulin.

*(B) Quantification of APP protein levels was calculated by normalising APP to β-tubulin values, which were obtained through quantitative densitometry. n=5. One-way ANOVA with Bonferroni's test for multiple comparisons, ***p=0.0001, ****p<0.0001.*

(C) DIV12-16 cortical neurons were transduced with lentivirus molecular replacement constructs and returned to conditioned media for 4 days. Cells were then lysed and immunoblotted for myc and β-tubulin.

(D) Quantification of myc protein levels was calculated by normalising myc to β-tubulin values, which were obtained through quantitative densitometry. n=1.

(E) Samples were run on 8% gel to demonstrate the difference in molecular weight between endogenous and recombinant protein, caused by the presence of the myc tag.

Figure 5-3 B demonstrated that cells expressing APP shRNA showed a dramatic reduction in endogenous APP expression. Both Shres-APP_{WT} and Shres-APP_{SWE} rescued APP protein expression to endogenous levels since APP densitometry was not significantly different from Scr control levels. Myc bands confirmed the replacement of endogenous APP with myc-tagged APP_{WT} or APP_{SWE} (figure 5-3 D). Figure 5-3 E demonstrates that when samples were run on 8% gel there was a difference in molecular weight between endogenous and recombinant protein, providing an additional validation that endogenous APP was being knocked down and replaced with myc-tagged APP_{WT} or APP_{SWE}.

5.3.3 Replacing endogenous APP with APP_{SWE} causes a loss of total GluA2

The Tg2576 mouse expressing human APP with the double Swedish mutation ($APP_{K670N/M671L}$) has previously demonstrated deficits in LTP (Fryer *et al*., 2003), a loss of dendritic spine density (Lanz, Carter and Merchant, 2003) and cognitive impairments (Jacobsen *et al*., 2006). These deficits can be indicative of aberrant AMPAR recycling. Together with the evidence that synaptic weakening occurs in AD (Cullen *et al*., 1997; Walsh *et al*., 2002; Wang *et al*., 2002; Shanker *et al*., 2007, Shanker *et al*., 2008) and that LTD is facilitated in response to amyloidopathy (Shanker *et al*., 2008; Li *et al*., 2009), studying AMPAR degradation in a cell culture model expressing APP_{SWE} would be of interest since changes to their composition and properties at the post-synaptic membrane can significantly modify synaptic function and neuronal connectivity. Cortical neuronal cultures were infected with the molecular replacement constructs as before and lysed 4 days later before immunoblotting for GluA1 or GluA2.

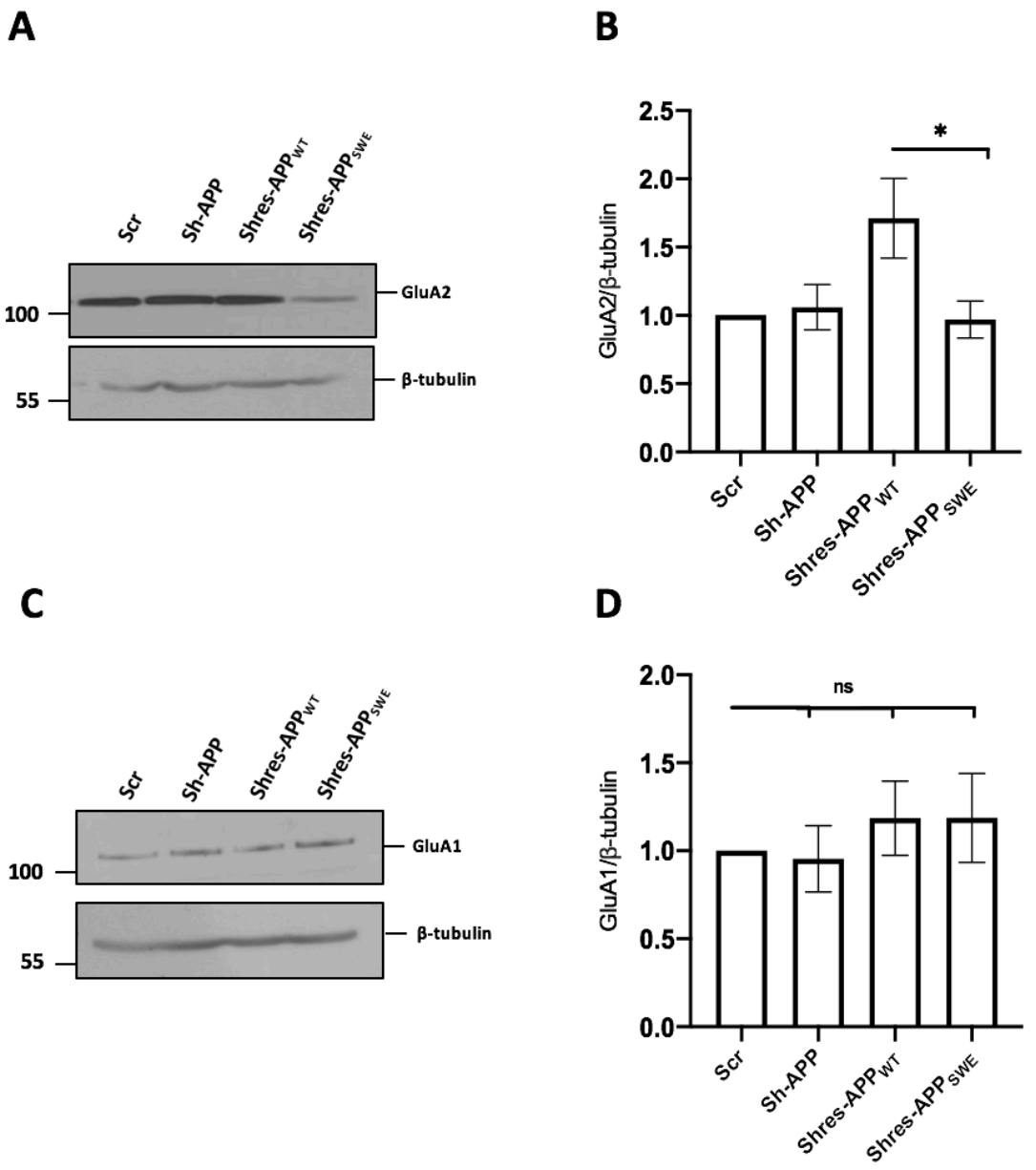

Figure 5-4 Knockdown of endogenous APP and replacement with APP_{SWE} in cortical neuronal cultures reduces total GluA2 expression.

DIV12-16 cortical neurons were transduced with lentiviral molecular replacement constructs and returned to conditioned media for 4 days. Cells were then lysed and immunoblotted using the indicated antibodies.

(A) Representative Western blots of GluA2 and ß-tubulin.

*(B) Quantification of GluA2 protein levels was calculated by normalising GluA2 to ß-tubulin values, which were obtained through quantitative densitometry. n=10-12. One-way ANOVA with Bonferroni's test for multiple comparisons, *p=0.0407.*

(C) Representative Western blots of GluA1 and β-tubulin.

(D) Quantification of GluA1 protein levels was calculated by normalising GluA1 to β-tubulin values, which were obtained through quantitative densitometry. n=10. One-way ANOVA with Bonferroni's test for multiple comparisons, p=0.7439.

Figure 5-4 B showed that knockdown of endogenous APP and replacement with APP_{SWE} decreased total GluA2 expression compared to APP_{WT} control cells. In contrast, figure 5-4 D demonstrated that GluA1 expression levels were not regulated by APP_{SWE} expression, since no significant difference was witnessed compared to APP_{WT} expression. Therefore, only total GluA2 levels were decreased in response to the APP Swedish mutant.

5.3.4 Validation of APP overexpression in J20 mouse models

The third AD model used to look at GluA1 and GluA2 expression was the J20 mouse model. J20 mice overexpress APP with both the Swedish ($APP_{K670N/M671L}$) and Indiana (APP_{V717F}) mutations. These mice should therefore demonstrate APP overexpression compared to controls, with the APP mutations responsible for the AD phenotypes. At 16-18 months of age, six J20 mouse brains and five WT mouse brains were snap frozen on dry ice and stored at -80°C before homogenisation and immunoblotting for GluA1 and GluA2.

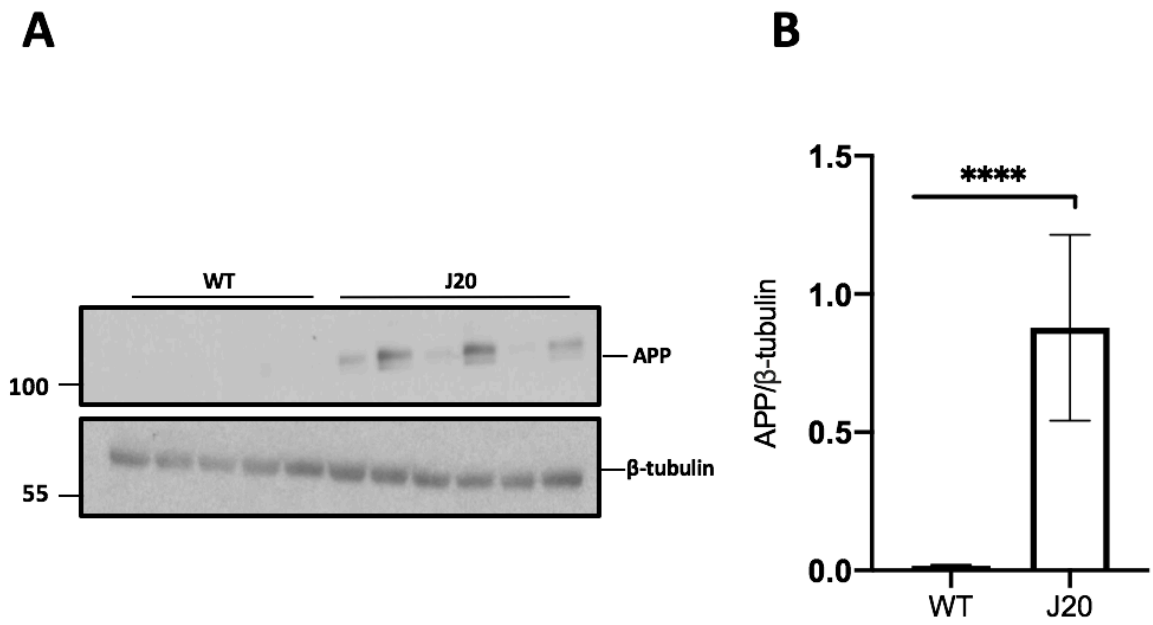

Figure 5-5 J20 mouse models overexpress APP.

(A) Five 16-18 month-old WT mouse brain and six 16-18 month old J20 mouse brains were homogenised and 7.5ug separated by SDS-PAGE and immunoblotted using the indicated antibodies.

*(B) Quantification of APP protein levels was calculated by normalising GluA2 to ß-tubulin values, which were obtained through quantitative densitometry. n=10-12. Mann Whitney test, ****p<0.0001.*

Figure 5-5 B demonstrated that APP was significantly overexpressed in J20 mouse brain compared to WT controls.

5.3.5 J20 mouse models demonstrate no change in GluA1 or GluA2 expression

The J20 mouse model has demonstrated deficits in LTP (Saganich *et al*., 2006) and cognitive impairments on the Morris water maze (Cheng *et al*., 2007) and radial arm maze (Wright *et al*., 2013). Furthermore, depletions in the expression of GluA1 and GluA2 have been observed in the dentate gyrus of J20 mice (Palop *et al*., 2007). We investigated whether or not a loss of GluA1 or GluA2 expression occurred in the cortex of J20 mouse brain. WT and J20 mouse brain were homogenised as before and immunoblotted for GluA1 or GluA2.

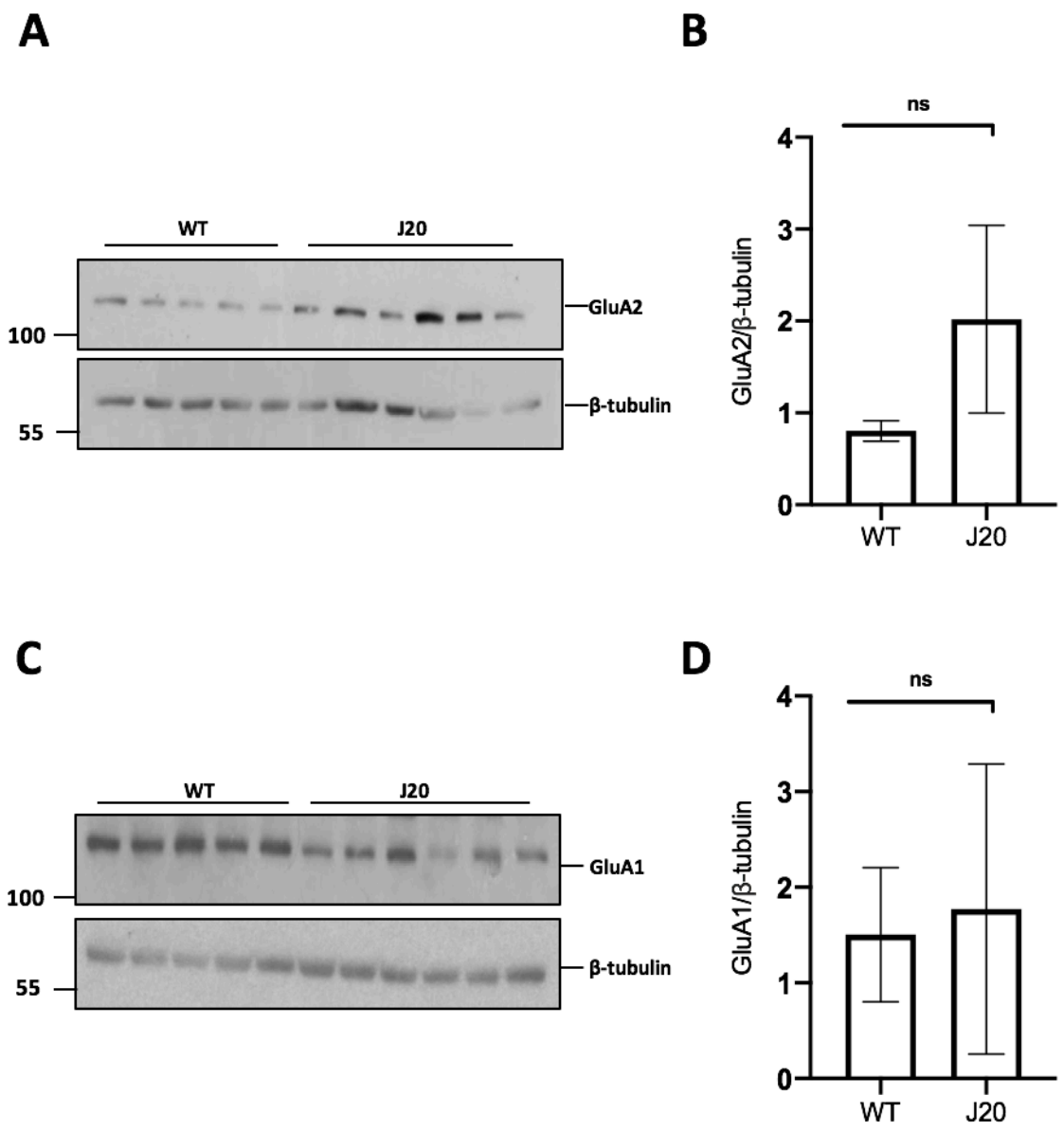

Figure 5-6 GluA1 and GluA2 subunit expression levels are not affected in J20 mouse models.

Five 16-18 month old WT mouse brain and six 16-18 month J20 mouse brains were homogenised and 7.5ug separated by SDS-PAGE and immunoblotted using the indicated antibodies.

(A) Representative Western blots of GluA2 and ß-tubulin.

(B) Quantification of GluA2 protein levels was calculated by normalising GluA2 to ß-tubulin values, which were obtained through quantitative densitometry. n=3. Paired two-tailed student's t-test, p=0.3884.

(C) Representative Western blots of GluA1 and ß-tubulin.

(D) Quantification of GluA1 protein levels was calculated by normalising GluA2 to ß-tubulin values, which were obtained through quantitative densitometry. n=3. Paired two-tailed student's t-test, p=0.7763.

Figure 5-6 B demonstrated that there was no significant change in GluA2 expression levels between J20 mouse models and WT controls. There was also no significant difference in GluA1 expression (figure 5-6 D). These results were surprising given the results from our other two models and from previous J20 mouse studies (Palop *et al*., 2007).

5.4 DISCUSSION

5.4.1 Reduced GluA2 expression following APP_{WT} overexpression and APP_{SWE} expression

Figure 5-1 B demonstrated that a decrease in total GluA2 expression was observed in response to APP_{WT} overexpression. This could not be seen with total GluA1 expression levels (figure 5-2 B). Similarly, figure 5-4 B demonstrated that total GluA2 expression was reduced in response to APP_{SWE} expression, but GluA1 expression was not affected (figure 5-4 D). Although a change to protein expression can indicate transcriptional down-regulation of protein messenger (m)RNA, it can also suggest changes in protein degradation (Goo, Scudder and Patrick, 2015). Given it has previously been demonstrated in the literature that both GluA1 and GluA2 are reduced in post-mortem AD brains compared to controls (Yasuda *et al*., 1995; Carter *et al*., 2004; Palop *et al*., 2007; Resende *et al*., 2007) it's possible that amyloidopathy could trigger the selective degradation of GluA2-containing AMPARs. However, neuronal protein degradation pathways need to be examined before conclusions can be drawn on this.

The observation that GluA2 but not GluA1 receptor subunits exhibited reduced expression in both of the above models demonstrates that the disease phenotype did not result in the non-specific loss of all surface proteins and indicates that Aβ acts through a selective mechanism that is AMPAR subunit specific. It might be that these models of amyloidopathy do not capture the mechanisms responsible for GluA1 subunit changes in human post-mortem tissues. Indeed, it has been suggested that tau phosphorylation is required to act as an intermediate signalling molecule between Aβ initiation and eventual synaptic dysfunction, since Aβ-induced synaptic impairments are diminished by the expression of a tau construct that cannot be phosphorylated (Miller *et al*., 2014).

However, differential effects on AMPAR-subunits are not unusual and have been witnessed in other disease states including ischaemic brain injury (Dixon, Mellor and Hanley, 2009; Blanco-Suarez and Hanley, 2014) and amyotrophic lateral sclerosis (Kawahara *et al*., 2004; Kwak *et al*., 2010). Given that

the majority of AMPARs in the mature hippocampus are made up of GluA1/2 or GluA2/3 heteromers (Wenthold *et al*., 1996; Lu *et al*., 2009), the results indicate that GluA2/3 receptors could be vulnerable to the effects of Aβ whilst GluA1/2 heteromers are protected by an unknown mechanism. A more recent study showing that the GluA3 subunit is important in the response to Aβ provides further support to this idea (Reinders *et al*., 2016).

A similar model for the specific loss of GluA2/3 heteromers has been proposed in response to ischaemia (Koszegi *et al*., 2017) however here, GluA2 down-regulation was observed in hippocampal but not cortical neurons and was thus dependent on a cell-type specific mechanism involving PICK1. In our experiments, a loss of GluA2 expression was demonstrated in both cortical and hippocampal *in vitro* models of AD, indicating the involvement of molecular cell biology that is common to both cell types.

5.4.2 APP_{WT} overexpression and AMPARs

In the past researchers have struggled to generate models of AD that overproduce Aβ without overexpressing APP, therefore in order to mimic Aβ pathology many research groups have utilised models that overexpress human WT APP (Kamenetz *et al*., 2003; Hsieh *et al*., 2006; Simón *et al*., 2009) or APP with human mutations (Gu, Liu and Yan, 2008; Cantanelli *et al*., 2014). However, increasing gene expression beyond physiological levels can introduce artefacts including the interference of protein homeostasis, which triggers apoptosis (Kuang *et al*., 2006). Thus, some researchers believe that APP overexpression is an artificial means to model AD. Indeed, it has been demonstrated that some phenotypes present in these models are unable to be reproduced in knock-in models of the disease, suggesting they are simply artefacts as a result of protein overexpression (Saito *et al*., 2014). However, it can also be argued that APP overexpression is not artificial since duplication of the entire APP gene causes early-onset familial forms of AD (McNaughton *et al*., 2012) and sufferers of Down's syndrome, who carry an extra copy of chromosome 21 that harbours the gene, develop the disease (García-Alba *et al*., 2019). Nevertheless, the possibility of artefacts in our disease model must be taken into account, but the fact the results in this model were replicated in our knock-in model of the disease suggests they are not simply a product of protein overexpression.

It was interesting to note that figure 5-1 A demonstrates all neurons in the field of view transfected with APP_{WT} had reduced GluA2 expression levels compared to control neurons. This suggests Aβ from the transfected neuron could be affecting AMPAR expression in neighbouring cells. Since Aβ is

secreted via exocytosis into the extracellular space, its affect on nearby cells isn't surprising (Bergmans and De Strooper, 2010).

Consistent with our results, a previous study has also demonstrated a loss of GluA2 receptor subunit expression in a mouse model overexpressing APP_{WT} (Simón *et al*., 2009). In contrast to our data however, this model also demonstrates total degradation of the GluA1 receptor subunit. Beside from the fact one system is overexpressing APP_{WT} in a transgenic mouse model and the other in a neuronal culture model, the inconsistencies could arise as a result of differences in the extent of Aβ build-up. Indeed, Simón and colleagues (2009) demonstrate that in both 5- and 8- month old APP_{WT} mice, barely detectable levels of $A\beta_{42}$ were present, which led to the conclusion that the pathological features in these mice were unrelated to the levels of Aβ. Thus, an Aβ-independent pathogenic pathway with mechanisms distinct from our model is probably to blame for the inconsistencies.

Few studies have directly investigated the consequences of overexpressing human APP_{WT} in neuronal cell culture on AMPAR expression, except Hsieh *et al* (2006) who examined its effects on endogenous GluA1 and GluA2 receptor subunit levels. It was established that APP_{WT} overexpression did not significantly affect endogenous GluA1 or GluA2 expression levels, a surprising contrast to our results. However, the differences in conclusions between our work and the previous study may be explained by differences in methodology. Indeed, Hsieh *et al* (2006) used Sindbis viral infection to deliver the APP constructs into organotypic slices, whereas we used lipofection in dissociated neuronal cultures. Furthermore, whereas subunit expression in our model was measured 48 hours post-transfection, Hsieh and colleagues measured total expression levels just 22-26 hours post-transduction (Hsieh *et al*., 2006). Perhaps Aβ build-up at this earlier time-point was not yet sufficient to significantly disrupt the trafficking of GluA2/3 heteromers towards lysosomal degradation. Examining AMPAR expression at a series of intervals would help determine how long after APP_{WT} transfection we can begin to see changes in subunit expression levels, in order to establish the time-course within which this phenomenon occurs.

5.4.3 APP_{SWE} expression and AMPARs

Figure 5-3 B demonstrated that endogenous APP could be successfully knocked down in cortical neuronal cultures using rat APP shRNA, and both APP_{WT} and APP_{SWE} could rescue protein expression. Since APP densitometry was not significantly different from Scr control levels, this indicated that the viruses developed by our lab rescued protein expression to endogenous levels, thus any artefacts

that could arise as a result of protein overexpression were prevented (Saito *et al*., 2014). All detectable APP in the APP_{WT} and APP_{SWE} replacement cultures was recombinant (figure 5-3 E).

Although our model demonstrated endogenous expression of the APP mutant APP_{SWE}, the expression levels of Aβ were not measured. However, previous evidence demonstrates that accumulation of Aβ is most likely responsible for AD phenotypes in this model (Almeida *et al*., 2005). Indeed, it has been demonstrated that cultured neurons from Tg2576 mice, which express the APP_{SWE} double mutation, exhibit a significant increase in Aβ levels (Takahashi *et al.,* 2004) and it has also been demonstrated that inhibition of both BACE1 and γ-secretase, which block the generation of Aβ, reverse AD phenotypes in Tg2576 mice (Ohno *et al*., 2004; Almeida *et al*., 2005). It is therefore likely that Aβ accumulation is responsible for the changes in AMPAR expression observed in our model, however, because this was not directly measured it cannot be assumed.

GluA2 levels did not significantly differ between the Scr control and Sh-APP conditions which is to be expected. Indeed, since under healthy conditions the majority of APP is cleaved by α- followed by γ-secretase in the non-amyloidogenic pathway (O'Brien and Wong, 2011), knocking down APP would not significantly affect these negligible levels of Aβ. It was, however, unexpected that the Shres-APP_{WT} condition demonstrated increased GluA2 expression in comparison to the Scr control and Sh-APP conditions; this was surprising given there was no overexpression of APP in the three conditions and therefore no increase in APP amyloidogenic processing. It could be that the myc tag on APP has some uncharacterised effect on AMPARs, perhaps indirectly via some other aspect of synaptic organisation. Alternatively, since the myc tag is on the c-terminus of APP, it could influence the activity of downstream APP cleavage products which might influence GluA2 receptor trafficking. Indeed, APP cleavage products have demonstrated the ability to modulate neuronal function and circuit activity (Harris *et al*., 2020).

AMPAR expression levels have not previously been explored in neuronal cultures directly infected with APP_{SWE}, but there is a study investigating subunit expression in a Tg2576 mouse model. Consistent with our results, Almeida *et al* (2005) demonstrated that DIV12 cultured neurons from Tg2576 mice exhibit no change in total levels of the GluA1 receptor subunit. This supports the idea that GluA1/2 heteromers are protected from the effects of Aβ, although GluA2 receptor subunit data is needed to confirm this.

5.4.4 J20 mice and AMPARs

Figure 5-6 B and D demonstrated that J20 mouse models showed no significant change in GluA1 or GluA2 expression levels compared to WT control mice. Since J20 mice express both the Indiana (APP_{V717F}) and the double Swedish mutation ($APP_{K670N/M671L}$), this model is associated with severe disease pathology (Saganich *et al*., 2006; Cheng *et al*., 2007; Wright *et al*., 2013), thus it is surprising that a loss of GluA2 expression is not witnessed in the disease state.

To the best of our knowledge, only one study to date has examined the expression of AMPARs in J20 mouse models. Here, it was established that levels of both GluA1 and GluA2 were reduced in the dentate gyrus of J20 mice compared to controls (Palop *et al*., 2007). The discrepancy between our results and the results of the previous study could be due to a number of fundamental differences in the methodology. Indeed, Palop and colleagues (2007) examined mice that were sacrificed at 4-7 months of age, whereas the mice we investigated were 16-18 months of age. However, this is unlikely to account for the observed differences since firstly, robust Aβ plaques develop in J20 models at 5 to 7 months of age meaning this pathological hallmark should be established in both sets of mice (Mucke *et al*., 2000); and secondly, a loss of subunit expression was witnessed in the younger mice, so it would be expected that the same phenotype, if not worse, would be observed in those at 16-18 months of age. The most likely explanation for the differences in the two sets of data is that Palop and colleagues (2007) examined subunit expression specifically in the dentate gyrus of the hippocampus. Given disruption to AMPAR trafficking has been widely demonstrated in this brain region (Almeida *et al* 2005; Hsieh *et al*., 2006; Simón *et al*., 2009; Megill *et al*., 2015) this might be why we didn't witness any changes in our model of the cortex.

Having said this, results from our previous two models suggest that the molecular mechanisms responsible for GluA2 down-regulation are not cell-type specific and are observed in both hippocampal and cortical neuronal cultures. Thus, an alternative explanation could be due to the differences in APP overexpression witnessed between the six J20 mouse brains. Although figure 5-5 B demonstrates that there is a significant increase in APP expression in J20 models compared to WT controls as a whole, there is a huge variation in the levels of APP overexpression between brains. Indeed, only two J20 mice demonstrate dramatic APP overexpression, with two mice exhibiting negligible overexpression. It is unclear why there is such a variation in the levels of APP overexpression between the mice, since there were all males and housed in the same conditions. It could be that we needed to optimise the brain homogenisation process. Although Aβ levels were not measured here, it's likely that in mice exhibiting lower levels of APP overexpression, there is not a

significant build-up of Aβ. This might explain why changes to AMPAR subunit expression were not witnessed, since we believe that it is the build-up of Aβ that is responsible for disruption to receptor trafficking. It

Additionally, due to time constraints, we only had an n of 3 for our experiments so the fact that we did not witness any significant differences could be because our experiment was underpowered, especially given the two-fold change in figure 5-6. More repeats would be needed in future investigations and it would also be interesting to analyse GluA2/GluA1 subunit ratios to show any potential changes here.

This chapter identified two models of AD in which expression of the GluA2 subunit was found to be reduced in the disease state. Considering reduced subunit expression can indicate changes to protein degradation, it was important to investigate how this might occur through the examination of neuronal protein degradation pathways.

6 GluA2 UNDERGOES LYSOSOMAL TARGETING IN RESPONSE TO APP OVEREXPRESSION

6.1 AIMS

Following the confirmation that GluA2 subunit expression was reduced in neuronal models of amyloidopathy, it was important to investigate whether this could be explained by degradation of GluA2-containing AMPARs. Thus, the trafficking of AMPARs to the lysosomal degradation pathway was examined in response to amyloidopathy. Potential molecular mechanisms responsible were also investigated by examining cortactin tyrosine phophorylation. It was hypothesised that APP_{WT} overexpression would lead to an increase in GluA2 lysosomal targeting, and APP_{SWE} expression would lead to an increase in cortactin tyrosine phosphorylation.

6.2 INTRODUCTION

It has been widely demonstrated that AMPARs are lost from the surface membrane in response to Aβ (Hsieh *et al*., 2006; Zhao *et al*., 2010; Alfonso *et al*., 2014). Some of the molecular mechanisms elucidated thus far suggest Aβ promotes surface receptor loss via an increase in AMPAR endocytosis (Hsieh *et al*., 2006; Almeida *et al*., 2005). However, another mechanism that can result in receptor loss from the surface is a disruption to trafficking so that instead of being recycled, receptors are targeted to lysosomes where they are subsequently degraded. Indeed, once internalised, receptors are trafficked into early endosomes (EEs) from which they can: undergo recycling to the post-synaptic membrane via recycling endosomes (REs) (van der Sluijs and Hoogenraad, 2011); mature into late endosomes (LEs) and fuse with lysosomes for degradation (Hu *et al*., 2015); or traffic back to the *trans*-Golgi network for further PTMs (Burd and Cullen, 2014). A complement of proteins and PTMs influence the endosomal sorting and fate of internalised receptors. These can be dysregulated in the disease state leading to aberrant lysosomal targeting of crucial synaptic proteins (Parkinson and Hanley, 2018).

Recent evidence has demonstrated that cortactin maintains surface levels of GluA2/3 heteromers by directing receptors away from lysosomes (Parkinson *et al*., 2018). In the absence of a cortactin-

GluA2 interaction, GluA2-containing AMPARs were targeted for lysosomal degradation. The interaction was negatively regulated by Src family kinase (SFK) phosphorylation of cortactin at tyrosine residues Y421 and Y466 (Parkinson *et al*., 2018).

Importantly, emerging evidence has identified a link between Aβ-mediated toxicity and aberrant SFK activity, particularly the kinase Fyn. Indeed, *in vitro* application of Aβ has been shown to increase tyrosine phosphorylation of an array of proteins in various preparations (Luo *et al*., 1995; Bamberger *et al*., 2003; Grace and Busciglio, 2003) and slices from Fyn knockout mice are spared of Aβ-mediated toxicity (Lambert *et al*., 1998). Furthermore, APP transgenic mouse models have demonstrated that Fyn overexpression both accelerates the onset of cognitive impairment and promotes Aβ-induced synaptotoxicity, a phenomenon which can be rescued by Fyn ablation (Chin *et al*., 2004, Chin *et al*., 2005).

Based on the knowledge that increased SFK cortactin phosphorylation promotes GluA2 lysosomal targeting (Parkinson *et al*., 2018) and aberrant SFK activity has been reported in response to Aβ expression (Luo *et al*., 1995; Bamberger *et al*., 2003; Grace and Busciglio, 2003), it was hypothesised that GluA2/3 heteromers might be targeted to the lysosome in response to amyloidopathy. Furthermore, it was hypothesised that increased tyrosine phosphorylation of cortactin might be a molecular mechanism involved in GluA2/3 lysosomal targeting.

Further interest for the lysosomal degradation of GluA2-containing AMPARs in AD came from the evidence that oligomeric Aβ can mimic or facilitate the induction of LTD (Hsieh *et al*., 2006; Shanker *et al*., 2008; Li *et al*., 2009). Indeed, since the sorting of internalised AMPARs to the lysosome is a key determinant in LTD induction (He *et al*., 2009; Fernández-Monreal *et al*., 2012), this suggests aberrant GluA2 lysosomal targeting may prevail in models of amyloidopathy and contribute to AMPAR expression loss. Additionally, it has previously been demonstrated that incubation of hippocampal neurons with Aβ results in increased expression of the E3 ligase Nedd4-1 (Zhang *et al*., 2018) and its recruitment to dendritic spines (Rodrigues *et al*., 2016). Since the ubiquitin system can regulate the fate of AMPARs by promoting degradation via the lysosome (Claque and Urbé, 2010) this provides additional evidence that AMPAR lysosomal degradation could be responsible for the loss of GluA2 expression, although proteasomal degradation is also a possibility here.

Experiments presented in this chapter investigated the lysosomal targeting of the AMPA receptor subunits GluA1 and GluA2 in response to amyloidopathy. The phosphorylation of cortactin at tyrosine residues Y421 and Y466 in response to amyloidopathy was also investigated in order to explore the molecular mechanism that might be responsible for GluA2/3 lysosomal targeting.

6.3 RESULTS

6.3.1 APP_{WT} overexpression promotes GluA2 lysosomal targeting

Antibody feeding assays are a powerful technique used to track the movement of internalised endogenous receptors through the endosomal pathway and have been successfully used before to define trafficking mechanisms in cultured neurons (Lee, Simonetta and Sheng, 2004; Koszegi, Fiuza and Hanley, 2017; Parkinson *et al.*, 2018). Thus, an antibody feeding assay was used to track the constitutive movement of GluA2-containing AMPARs to lysosomes by examining co-localisation between internalised GluA2 and the lysosomal marker LAMP1. In chapter 3, it was demonstrated that overexpressing APP_{WT} in hippocampal neuronal cultures resulted in reduced GluA2 expression. This made it an appropriate model to investigate the involvement of the lysosomal pathway in GluA2-containing AMPAR expression loss.

Experiments were carried out in hippocampal neuronal cultures transfected with APP_{WT} or APP_{MV} and returned to conditioned media for 20 or 45 minutes following antibody incubation to allow constitutive trafficking of surface labelled receptors through the endosomal system. The co-localisation of internalised GluA2 with LAMP1 was considered an indication of AMPAR lysosomal targeting.

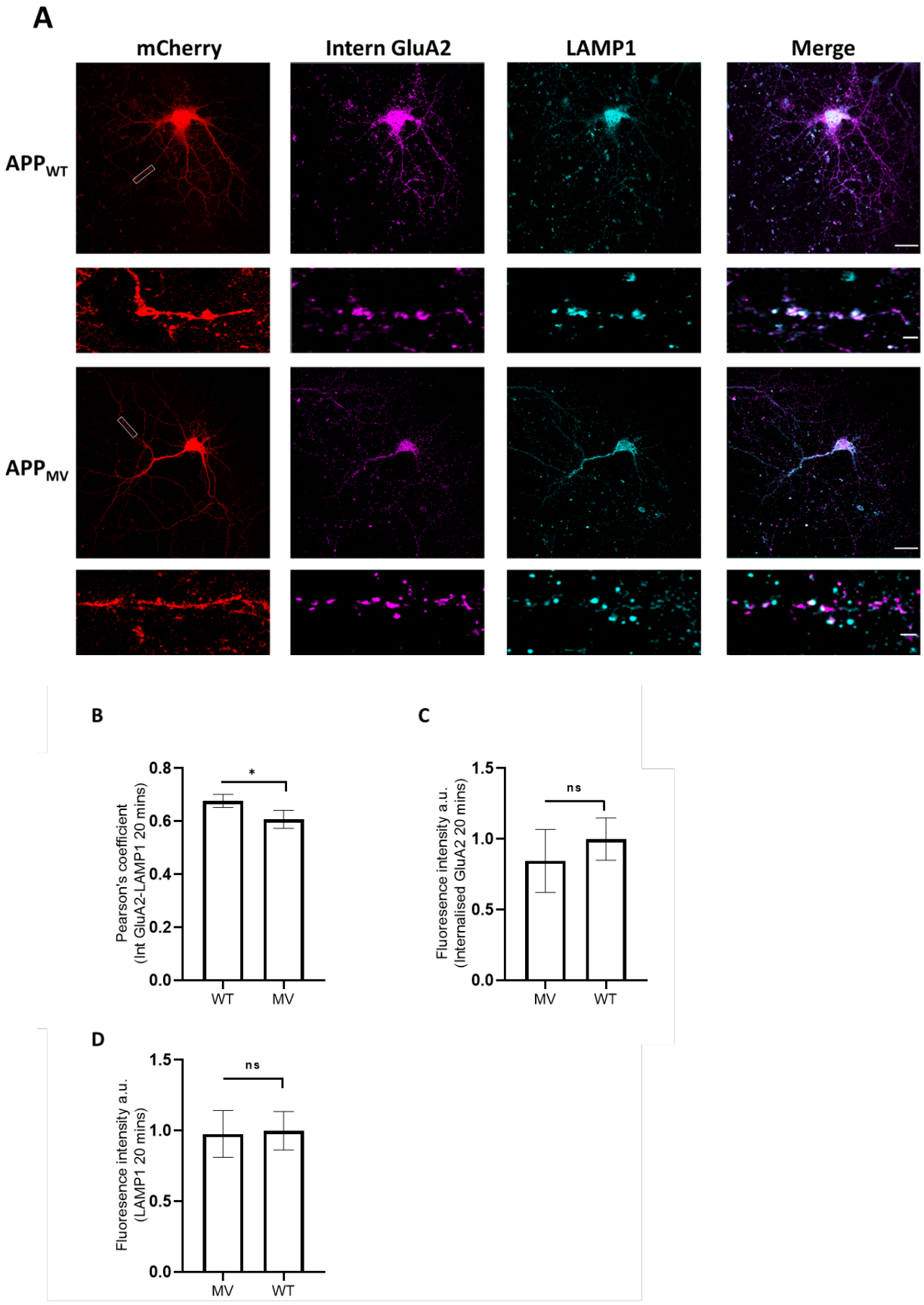

Figure 6-1 Overexpression of APP_{WT} results in GluA2 lysosomal targeting at 20 minutes.

(A) DIV13 primary hippocampal neurons were transfected with plasmids encoding APP_{WT}–IRES-mCherry or APP_{MV}-IRES-mCherry and returned to conditioned media for 24 hours. Cells were then live labelled for GluA2 and returned to conditioned media for 20 minutes to allow trafficking of surface labelled GluA2 through the endosomal system before co-localisation analysis was carried out between internalised GluA2 and LAMP1. Transfected cells were identified by mCherry fluorescence (red channel). The magenta channel represents staining for internalised GluA2 and the cyan channel for LAMP1. Full image scale bar=30µm. Panels below show magnification of a portion of dendrite in full images. Magnified panel scale bar=5µm.

*(B) Quantification of the co-localisation between LAMP1 and internalised GluA2 at 20 minutes. n=6. Wilcoxon matched-pairs signed rank test, *p=0.0313.*

(C) Quantification of mean fluorescence intensity of internalised GluA2 at 20 minutes in the same dendrites analysed in (B). n=6. Paired two-tailed student's t-test, p=0.5642.

(D) Quantification of mean fluorescence intensity of internalised LAMP1 at 20 minutes in the same dendrites analysed in (B). n=6. Wilcoxon matched-pairs signed rank test, p>0.9999.

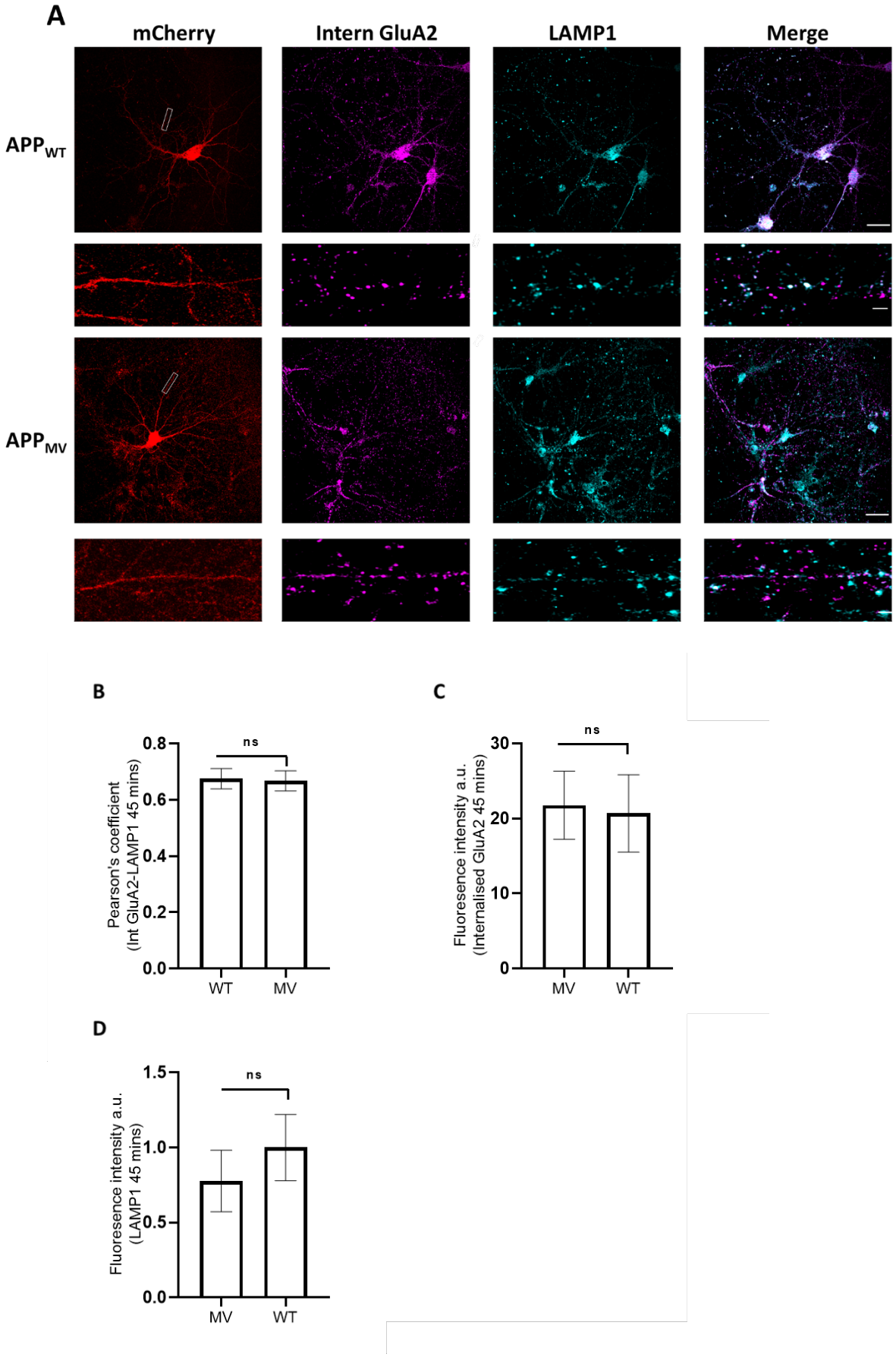

Figure 6-2 Overexpression of APP_{WT} does not result in GluA2 lysosomal targeting at 45 minutes.

(A) DIV13 primary hippocampal neurons were transfected with APP_{WT}-IRES-mCherry or APP_{MV}-IRES-mCherry and returned to conditioned media for 24 hours. Cells were then live labelled for GluA2 and returned to conditioned media for 45 minutes to allow trafficking of surface labelled GluA2 through the endosomal system before co-localisation analysis was carried out between internalised GluA2 and LAMP1. Transfected cells were identified by mCherry fluorescence (red channel). The magenta channel represents staining for internalised GluA2 and the cyan channel for LAMP1. Full image scale bar=30µm. Panels below show magnification of a portion of dendrite in full images. Magnified panel scale bar=5µm.

(B) Quantification of the co-localisation between LAMP1 and internalised GluA2 at 45 minutes. n=6. Wilcoxon matched-pairs signed rank test, p=0.8438.

(C) Quantification of mean fluorescence intensity of internalised GluA2 at 45 minutes in the same dendrites analysed in (B). n=6. Paired two-tailed student's t-test, p=0.2097.

(D) Quantification of mean fluorescence intensity of internalised LAMP1 at 45 minutes in the same dendrites analysed in (B). n=6. Wilcoxon matched-pairs signed rank test, p=0.4527.

Figure 6-1 B demonstrated that 20 minutes after internalisation from the cell surface, APP_{WT} overexpression caused a small but significant increase in the Pearson's correlation co-efficient between internalised GluA2 and LAMP1 compared to APP_{MV} control. These results suggest that APP_{WT} overexpression caused a loss of total GluA2 by increasing the lysosomal targeting of GluA2-containing AMPARs. 45 minutes after internalisation from the cell surface, the Pearson's correlation co-efficient was indistinguishable between the two conditions, indicating no difference in GluA2/LAMP1 co-localisation (figure 6-2 B). A likely explanation for this is that the GluA2 trafficked to lysosomes as a consequence of APP_{WT} expression had been subjected to lysosomal degradation between 20 and 45 minutes.

Despite the fact that Pearson's correlation coefficient is robust to differences in signal intensity (Dunn, Kamocka and McDonald, 2011), it was of interest to determine whether levels of internalised GluA2 or LAMP1 changed between the APP_{WT} and APP_{MV} conditions. Figure 6-1 C and D demonstrate no differences in signal intensity for either GluA2 or LAMP1 which further rules out the results being due to changes in intensities of either channel. These data are of additional importance because: internalised GluA2 intensity provides an indication of any change to endocytosis, which has been demonstrated in response to amyloidopathy and could influence trafficking to lysosomes (Hsieh *et al*., 2006); and LAMP1 intensity can indicate whether or not changes to lysosomes are occurring in response to Aβ, which has also been demonstrated (Knauer *et al*., 1992; Yang *et al*., 1998; Ditaranto, Tekirian and Yang, 2001; Liu *et al*., 2010b).

Total expression levels of GluA1 did not change in cells overexpressing APP_{WT} compared to APP_{MV}, which suggests there is no change in GluA1 subunit sorting to degradative pathways in response to Aβ. This was verified by investigating the effect of APP_{WT} overexpression on GluA1 lysosomal targeting. Hippocampal neurons were cultured, transfected and prepared for antibody feeding immunocytochemistry as before (figure 6-2 A). Figures 6-3 B and 6-4 B demonstrate that at both 20 minutes and 45 minutes there was no significant difference in Pearson's correlation coefficient between cells overexpressing APP_{WT} and APP_{MV}, suggesting no increase in lysosomal degradation of GluA1-containing AMPARs. Thus, APP_{WT} overexpression does not influence GluA1 trafficking to lysosomes.

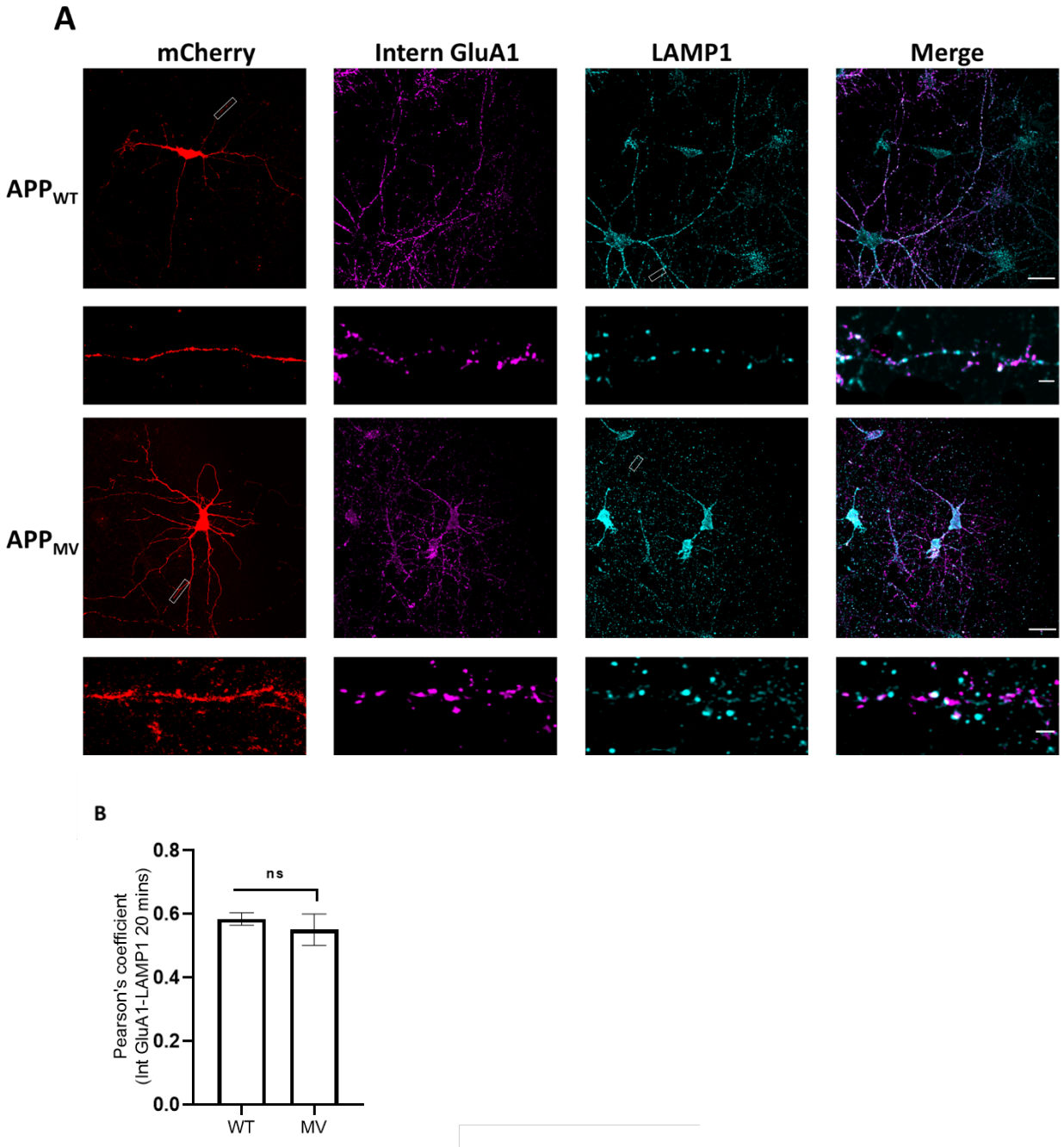

Figure 6-3 Overexpression of APP_{WT} does not result in GluA1 lysosomal targeting at 20 minutes.

(A) DIV13 primary hippocampal neurons were transfected with APP_{WT}-IRES-mCherry or APP_{MV}-IRES-mCherry and returned to conditioned media for 24 hours. Cells were then live labelled for GluA1 and returned to conditioned media for 20 minutes to allow trafficking of surface labelled GluA1 through the endosomal system before co-localisation analysis was carried out between internalised GluA1 and LAMP1. Transfected cells were identified by mCherry fluorescence (red channel). The magenta channel represents staining for internalised GluA1 and the cyan channel for LAMP1. Full image scale bar=30µm. Panels below show magnification of a portion of dendrite in full images. Magnified panel scale bar=5µm.

(B) Quantification of the co-localisation between LAMP1 and internalised GluA1 at 20 minutes. n=6. Wilcoxon matched-pairs signed rank test, p=0.8438.

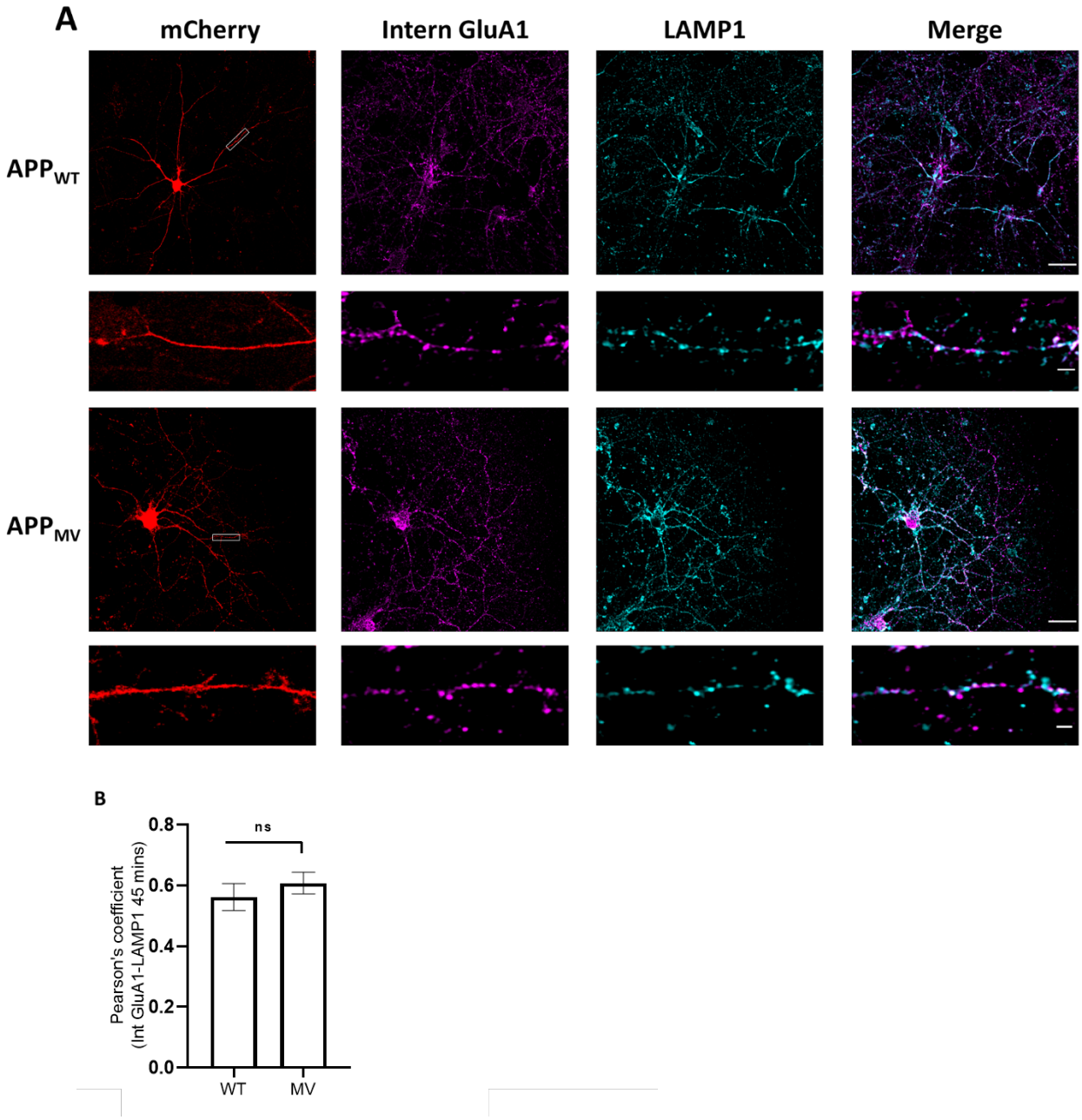

Figure 6-4 Overexpression of APP_{WT} does not result in GluA1 lysosomal targeting at 45 minutes.

(A) DIV13 primary hippocampal neurons were transfected with APP_{WT}-IRES-mCherry or APP_{MV}-IRES-mCherry and returned to conditioned media for 24 hours. Cells were then live labelled for GluA1 and returned to conditioned media for 45 minutes to allow trafficking of surface labelled GluA1 through the endosomal system before co-localisation analysis was carried out between internalised GluA1 and LAMP1. Transfected cells were identified by mCherry fluorescence (red channel). The magenta channel represents staining for internalised GluA1 and the cyan channel for LAMP1. Full image scale bar=30µm. Panels below show magnification of a portion of dendrite in full images. Magnified panel scale bar=5µm.

(B) Quantification of the co-localisation between LAMP1 and internalised GluA1 at 45 minutes. n=6. Wilcoxon matched-pairs signed rank test, p=0.6875.

6.3.2 Leupeptin restores GluA2/LAMP1 co-localisation at 45 minutes after internalisation from the cell surface

GluA2/LAMP1 co-localisation was indistinguishable between cells overexpressing APP_{WT} and APP_{MV} at 45 minutes after internalisation from the cell surface. To test whether this was due to lysosomal degradation of GluA2 at this later time point, cultures were treated with leupeptin, a cell-permeable protease inhibitor that inhibits cysteine, serine and threonine peptidases (Chu *et al*., 1998). Treating cultures with leupeptin blocks lysosomal protein hydrolysis and therefore should prevent degradation of GluA2-containing AMPARs, restoring the increase in GluA2/LAMP1 co-localisation seen with APP_{WT} at 20 minutes. Experiments were carried out as before except hippocampal neuronal cultures were treated with 100 µg/ml leupeptin for 3 hours prior to and during GluA2 antibody incubation and trafficking.

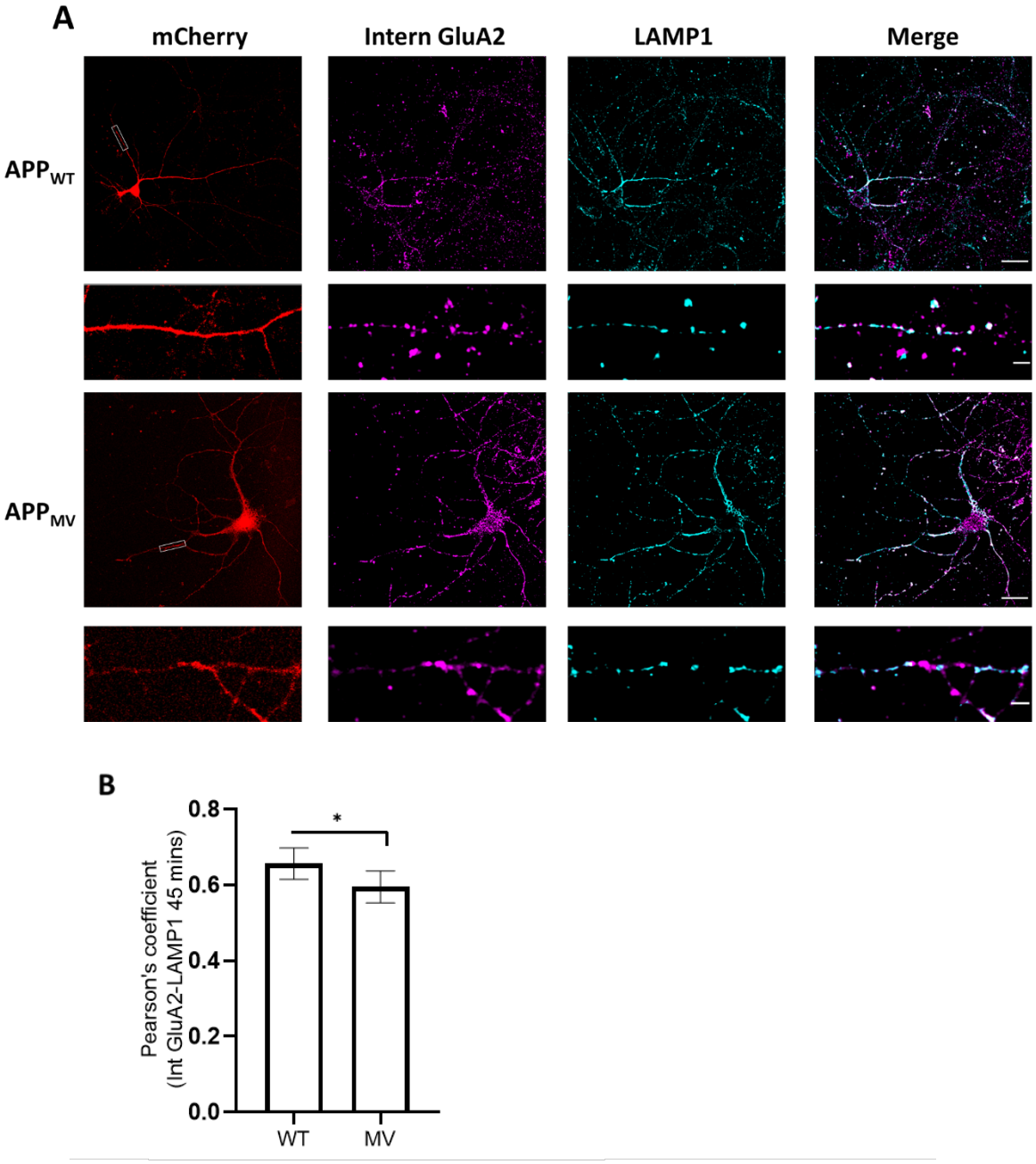

Figure 6-5 Leupeptin restores GluA2/LAMP1 co-localisation at 45 minutes after internalisation from the cell surface.

(A) DIV13 primary hippocampal neurons were transfected with APP_{WT}-IRES-mCherry or APP_{MV}-IRES-mCherry and returned to conditioned media for 21 hours. Cultures were treated with 100 µg/ml leupeptin 3 hours prior to and during primary antibody incubation and trafficking. Cells were live labelled for GluA2 and returned to conditioned media for 45 minutes to allow trafficking of surface labelled GluA2 through the endosomal system before co-localisation analysis was carried out between internalised GluA2 and LAMP1. Transfected cells were identified by mCherry fluorescence (red channel). The magenta channel represents staining for internalised GluA1 and the cyan channel for LAMP1. Full image scale bar=30µm. Panels below show magnification of a portion of dendrite in full images. Magnified panel scale bar=5µm.

*(B) Quantification of the co-localisation between LAMP1 and internalised GluA2 at 45 minutes in the presence of leupeptin. n=6. Wilcoxon matched-pairs signed rank test, *p=0.0313.*

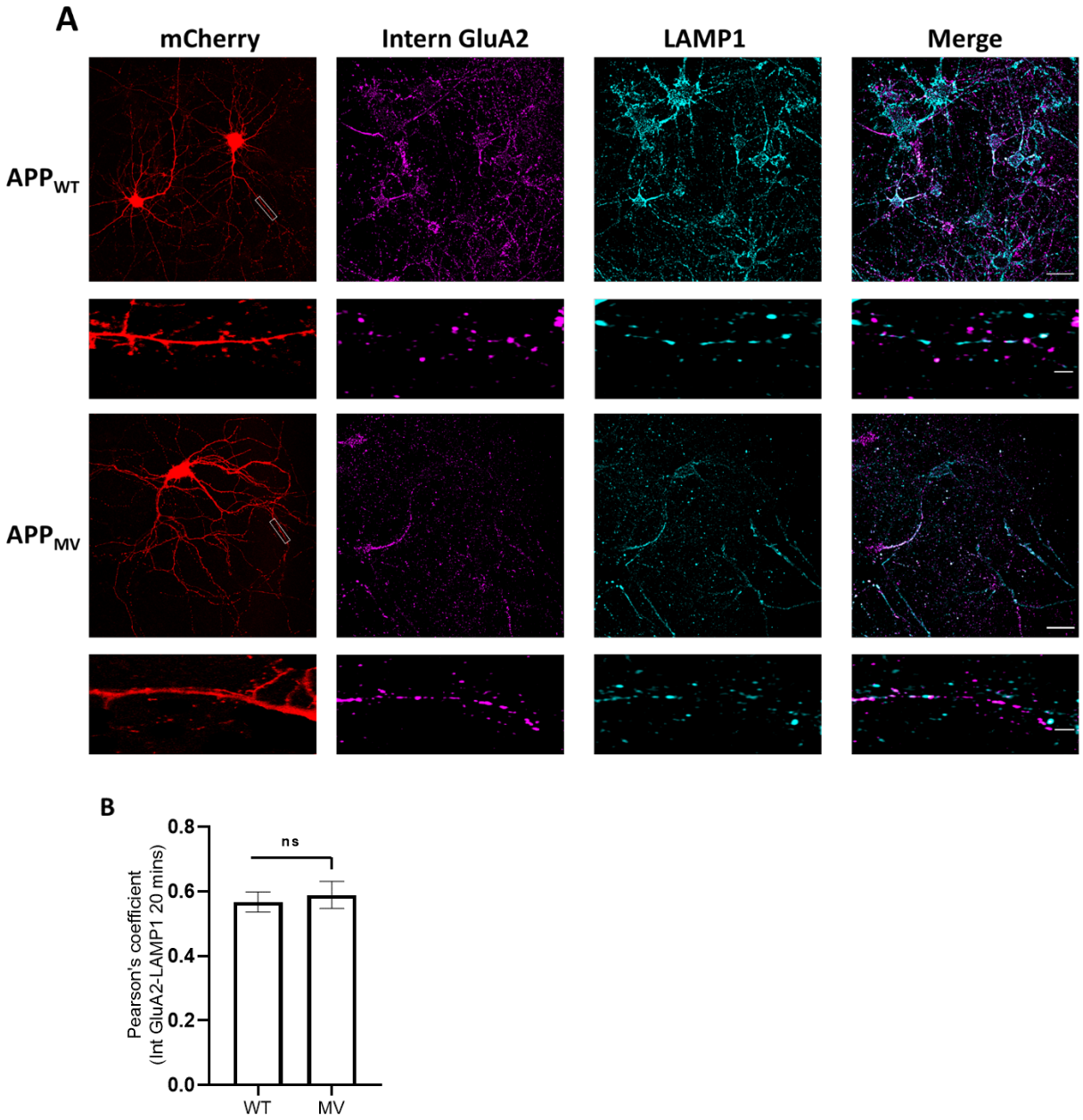

Figure 6-6 Leupeptin treatment abolishes GluA2/LAMP1 co-localisation at 20 minutes after internalisation from the cell surface.

(A) DIV13 primary hippocampal neurons were transfected with APP_{WT}-IRES-mCherry or APP_{MV}-IRES-mCherry and returned to conditioned media for 21 hours. Cultures were treated with 100 µg/ml leupeptin 3 hours prior to and during primary antibody incubation and trafficking. Cells were live labelled for GluA2 and returned to conditioned media for 20 minutes to allow trafficking of surface labelled GluA2 through the endosomal system before co-localisation analysis was carried out between internalised GluA2 and LAMP1. Transfected cells were identified by mCherry fluorescence (red channel). The magenta channel represents staining for internalised GluA1 and the cyan channel for LAMP1. Full image scale bar=30µm. Panels below show magnification of a portion of dendrite in full images. Magnified panel scale bar=5µm.

(B) Quantification of the co-localisation between LAMP1 and internalised GluA2 at 20 minutes in the presence of leupeptin. n=6. Wilcoxon matched-pairs signed rank test, p=0.5625.

Figure 6-5 B demonstrated that in the presence of leupeptin, at 45 minutes after receptor internalisation from the cell surface, APP_{WT} overexpression caused an increase in the Pearson's correlation co-efficient between internalised GluA2 and LAMP1. Thus, leupeptin restored the increase in GluA2/LAMP1 co-localisation, supporting the finding that APP_{WT} overexpression causes degradation of GluA2-containing AMPARs in lysosomes between 20 and 45 minutes. Surprisingly, leupeptin abolished the increase in Pearson's correlation co-efficient at 20 minutes that was observed previously following APP_{WT} overexpression (figure 6-6 B). This suggests leupeptin might have off target effects in slowing down receptor internalisation and trafficking through the endosomal system so that at 20 minutes, GluA2-containing AMPARs were not yet co-localised with lysosomes.

6.3.3 Knockdown of endogenous APP and replacement with APP_{SWE} causes an increase in cortactin Y466 phosphorylation

Overexpression of APP_{WT} demonstrated that GluA2-containing AMPARs were selectively targeted to lysosomes in response to amyloidopathy. It was therefore of interest to elucidate the molecular mechanisms responsible. Cortactin can be phosphorylated on tyrosine residues 421, 466 and 482 by SFKs, which regulate its ability to bind interacting proteins (Huang *et al*., 1997a; Dudek *et al*., 2002; Martinez-Quiles *et al*., 2004; Zhu *et al*., 2007). Importantly, the phosphorylation of cortactin at residues 421 and 466 negatively regulates the cortactin-GluA2 interaction resulting in the targeting of GluA2-containing AMPARs for lysosomal degradation (Parkinson *et al*., 2018). Since enhanced SFK activity has been widely demonstrated in AD (Shirazi and Wood, 1993; Lambert *et al*., 1998; Chin *et al*., 2004; Chin *et al*., 2005) it was of interest to investigate the tyrosine phosphorylation of cortactin in response to amyloidopathy.

Although lysosomal targeting of GluA2 was demonstrated in APP_{WT} overexpression cultures, due to time constraints the molecular replacement constructs were chosen for further investigation of GluA2 degradative mechanisms. It was demonstrated in chapter 3 that the replacement of endogenous APP with APP_{SWE} resulted in reduced GluA2 expression, therefore this was an appropriate model to investigate possible molecular mechanisms leading to GluA2-containing AMPAR degradation. Western blotting could be used as an effective technique to detect phosphorylated levels of cortactin, and the phospho-specific antibodies for tyrosine 421 and 466 of

cortactin had previously been validated (Zhou *et al*., 2006b; Tehrani *et al*., 2007; Sánchez, Urrego and Pardo, 2016; Parkinson *et al*., 2018). Cortical neuronal cultures were infected with the molecular replacement constructs as before and lysed 4 days later, followed by immunoblotting for pY421- and pY466- cortactin.

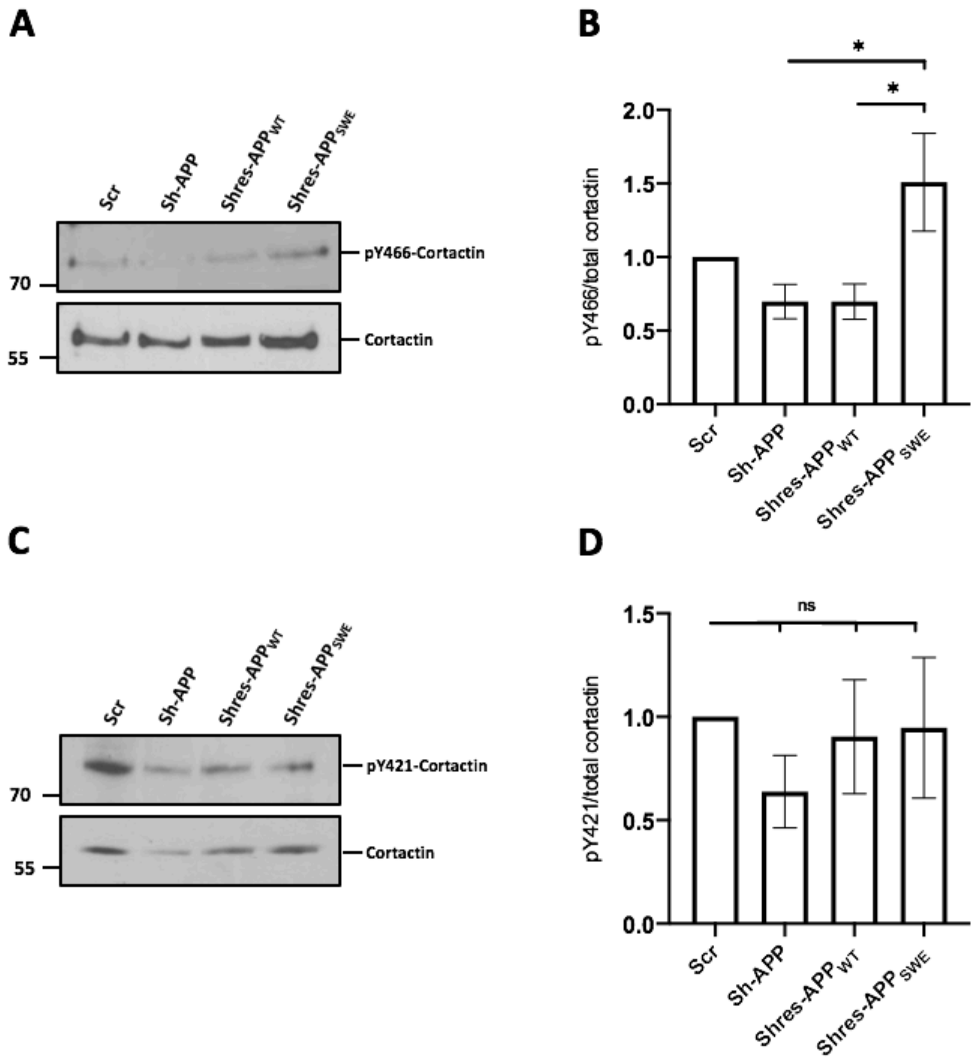

Figure 6-7 Knockdown of endogenous APP and replacement with APP_{SWE} in cortical neuronal cultures causes a specific increase in pY466-cortactin phosphorylation.

DIV12-16 cortical neurons were transduced with lentiviral molecular replacement constructs and returned to conditioned media for 4 days. Cells were then lysed and immunoblotted using the indicated antibodies.

(A) Representative Western blots of pY466-cortactin and total cortactin.

*(B) Quantification of cortactin phosphorylation at Y466. Band intensities were obtained through quantitative densitometry and pY466-cortactin values were normalised to total cortactin. n=7. One-way ANOVA with Bonferroni's test for multiple comparisons, *p=0.0308.*

(C) Representative Western blots of pY421-cortactin and total cortactin.

(D) Quantification of cortactin phosphorylation at Y421. Band intensities were obtained through quantitative densitometry and pY421-cortactin values were normalised to total cortactin. n=7. Kruskal-Wallis with Dunn's test for multiple comparisons, p=0.3567.

Figure 6-7 B demonstrated that cortactin phosphorylation at Y466 was increased in response to APP_{SWE}. In contrast, cortactin phosphorylation at Y421 was not significantly affected by knockdown of endogenous APP and replacement with APP_{SWE} compared to APP_{WT} control cells (figure 6-7 D). Therefore, modelling amyloidopathy led to the selective increase in cortactin phosphorylation at tyrosine 466.

6.4 Discussion

6.4.1 Lysosomal targeting of GluA2-containing AMPARs

Figure 6-1 B showed that overexpression of amyloidogenic APP in hippocampal neuronal cultures resulted in increased co-localisation between GluA2 and LAMP1 within 20 minutes, which strongly suggested that the trafficking of GluA2-containing AMPARs to the lysosome was responsible for their reduced expression. Analysis of the co-localisation at 45 minutes strengthened the conclusion that GluA2-containing receptors were indeed being degraded, since the increased co-localisation observed at 20 minutes returned to baseline levels at 45 minutes (figure 6-2 B). The timing for AMPARs to reach lysosomal compartments was quicker than the trafficking to lysosomes under basal conditions (Parkinson *et al.*, 2018), but similar to what has been established after bath application of AMPA and NMDA (Ehlers, 2000; Lee, Simonetta and Sheng, 2004), which was to be expected.

No change in GluA1/LAMP1 co-localisation was observed between APP_{WT} and APP_{MV} (figure 6-3 B, figure 6-4 B), which was to be expected since it was previously demonstrated in figure 5-2 that there was no significant decrease in GluA1 expression levels in this model of amyloidopathy. Given that

the majority of AMPARs in the mature hippocampus are made up of GluA1/2 or GluA2/3 heteromers (Wenthold *et al*., 1996; Lu *et al*., 2009), this further supports the view that it is GluA2/3 heteromers that are targeted for lysosomal degradation in the disease state. Additional support for this model comes from a report suggesting that the GluA3 subunit promotes lysosomal targeting (Lee, Simonetta and Sheng, 2004) and evidence that mice lacking GluA3-containing AMPARs are protected against Aβ-induced synaptic deficits (Reinders *et al*., 2016). Furthermore, a study screening for gene-expression profiles associated with mild cognitive impairment, a transitional stage between aging and AD, highlighted GluA3 as having a strong negative correlation with cognitive performance (Berchtold *et al*., 2014). Indeed, it may be the case that the GluA3 subunit is predominantly responsible for GluA2/3 lysosomal sorting. This would be worth studying in future investigations.

Although significant co-localisation was witnessed between internalised GluA2 and LAMP1 within 20 minutes, the difference in mean Pearson's co-efficient between cells overexpressing APP_{WT} and APP_{MV} cells, and thus the overall effect size, was small. This could suggest that although a significant portion of GluA2-containing AMPARs are targeted for lysosomal degradation in response to Aβ, this pathway is not solely responsible for the loss of GluA2 expression levels. There are a number of reasons why this could be.

There exists a body of evidence that describes abnormalities in the endolysosomal network in AD. Indeed, a striking neuronal pathology in the disease is the presence of autolysosomes contained within giant neuritic swellings, which suggests the elimination of substrates from these lysosomes is defective (Nixon *et al*., 2005). $A\beta_{42}$ aggregates can accumulate in lysosomes, resulting in disruption of the endolysosomal pathway (Knauer *et al*., 1992; Ditaranto, Tekirian and Yang, 2001; Liu *et al*., 2010b), disruption to the lysosomal membrane proton gradient (Ditaranto, Tekirian and Yang, 2001) and a loss of lysosomal membrane impermeability which precedes cell death (Yang *et al*., 1998). Since we modelled amyloidopathy in our experiments, $A\beta_{42}$ aggregates could have resulted in lysosomal disruption and contributed to the small effect-size of GluA2-LAMP1 co-localisation.

It must be noted that in the early stages of AD, lysosomal biogenesis is actually up-regulated in response to rab5-mediated acceleration of endocytosis (Cataldo *et al*., 1995; Bordi *et al*., 2016) as evidenced by growth of the lysosomal population, activation of transcription factors that regulate lysogenesis and the expression of lysosomal gene targets (Nixon and Cataldo, 2006; Bordi *et al*., 2016). Indeed, it is not until later on that lysosomes become dysfunctional, as reflected by their enlargement as they accumulate endocytic substrates (Nixon, 2017). However, in our model there appeared to be no change in LAMP1 expression levels in APP_{WT} neurons compared to controls (figure 6-1 D, figure 6-2 D) indicating that lysosomal biogenesis was not exhibited here.

AMPAR degradation occurs not only via endolysosomal trafficking, but through a variety of other mechanisms including the ubiquitin proteasome system (UPS) (Goo, Scudder and Patrick, 2015). Indeed, polyubiquitination targets receptors for degradation by the 26S proteasome (Claque and Urbé, 2010). Thus proteosomal degradation could also be responsible for the loss of GluA2 expression. In order to investigate this, GluA2 expression levels could be examined after treating neuronal cultures with a proteasome inhibitor such as MG-132, which blocks the proteolytic activity of the 26S proteasome complex (Han *et al*., 2009). Indeed, if GluA2-containing AMPARs were being degraded via the UPS, treatment with MG-132 would result in a smaller effect size for the loss of GluA2 expression. Furthermore, if lysosomal and proteosomal degradation were solely responsible for the loss of GluA2 expression in this model of amyloidopathy, treating cultures with both MG-132 and leupeptin would result in the loss of expression being eradicated. Since both of these systems operate downstream of AMPAR ubiquitination, which is upregulated in the disease state (Rodrigues *et al*., 2016; Guntupalli *et al*., 2017; Zhang *et al*., 2018), the involvement of both of these degradation pathways is likely.

However, there is also evidence of proteasome dysfunction in AD pathogenesis. Indeed, a reduction in proteasome activity was observed in post-mortem brains from AD patients compared to control brains, primarily in regions crucial for long-term memory including the hippocampus and parahippocampal gyrus (Keller, Hanni and Markesbery, 2001), and *in vitro* evidence demonstrates that $A\beta_{42}$ can inhibit proteasome activity to the same extent as the proteasomal inhibitor lactacystin (Oh *et al*., 2005). These findings should be taken into consideration when examining the involvement of proteasome degradation in the loss of GluA2 expression.

6.4.2 Leupeptin and lysosomal sorting

Leupeptin is a cell-permeable protease inhibitor that inhibits cysteine, serine and threonine peptidases (Chu *et al*., 1998) and thus should prevent the degradation of GluA2-containing AMPARs. Figure 6-5 B demonstrated that application of leupeptin restored GluA2/LAMP1 co-localisation at 45 minutes, which helped to confirm GluA2-containing AMPARs were indeed being degraded. Surprisingly however, leupeptin abolished the increase in co-localisation at 20 minutes (figure 6-6 B). It's possible that leupeptin could have off target effects that slow down receptor internalisation or sorting, so that at 20 minutes GluA2/3 heteromers were not yet co-localised with lysosomes. There is little literature examining the effects of leupeptin on receptor endocytosis and sorting mechanisms, but previous evidence in mammalian cells has demonstrated a decrease in the amount

of endocytosed ferritin ending up in lysosomes at various timepoints in leupeptin treated cells compared to controls (Miki and Kugler, 1986). Thus, it's possible that trafficking in the endosomal pathway could have been affected by leupeptin in this experiment. It would be useful to employ a different inhibitor of lysosomal degradation such as bafilomycin which inhibits vaculolar-type H+-ATPases (Tapper and Sundler, 1995) to determine whether or not co-localisation at 20 minutes remains and whether the results are due to leupeptin interfering with endosomal trafficking.

6.4.3 Aβ and endocytosis

Figure 6-1 C and 6-2 C demonstrated that there was no difference in levels of internalised GluA2 between cells overexpressing APP_{WT} and control cells at both 20 and 45 minutes. This suggests that GluA2 endocytosis per se is unaffected by Aβ. Other studies, however, have demonstrated an increase in AMPAR endocytosis in response to Aβ. Indeed, Hsieh *et al* (2006) demonstrated that when a GluA2 mutant that couldn't undergo AP2-mediated endocytosis was co-expressed with APP_{WT}, AMPA-mediated transmission was no different to cells expressing APP_{MV}. This indicated that Aβ produced by APP overexpression induced synaptic depression by driving endocytosis of AMPARs. It's likely that the difference in conclusions between our work and the previous study are due to the fact the GluA2 mutation that prevents AP2-mediated endocytosis is in the same region of the protein that binds cortactin. Thus, the results could be explained by an occlusion as a result of disrupting GluA2-cortactin binding. Furthermore, there were marked differences in the methodology used. Indeed, we directly tested GluA2 internalisation using antibody feeding assays whereas Hsieh *et al* measured the amplitude and inward rectification of evoked AMPAR transmission (Hsieh *et al*., 2006).

Several studies have demonstrated that application of $A\beta_{40}$ and $A\beta_{42}$ to hippocampal neuronal cultures reduce surface expression of GluA2 though a mechanism that is dependent on PICK1. Indeed, an increase in GluA2 phosphorylation at Ser-880 has been demonstrated (Liu *et al*., 2010a), which weakens its interaction with GRIP and allows PICK1 to bind (Seidenman *et al*., 2003). Similarly, Alfonso *et al* (2014) demonstrated a loss of surface GluA2 following treatment of CT100, the precursor to Aβ, that was dependent on an interaction with PICK1. Since PICK1 has demonstrated involvement in both the endocytosis and endosomal sorting of GluA2-containing AMPARs (Lu and Ziff, 2005; Sossa, Court & Carroll, 2006; Lin and Huganir, 2007; Widagdo *et al*., 2016; Fiuza *et al*., 2017; Koszegi, Fiuza and Hanley, 2017), it might be that both mechanisms contribute to the pathology of AD.

6.4.4 Cortactin phosphorylation in response to amyloidopathy

Figure 6-7 B demonstrated that cortactin phosphorylation at Y466 was increased in response to the expression of APP_{SWE}. Phosphorylation was specific to this tyrosine residue since Y421 phosphorylation was not increased in this model (figure 6-7 D). Y466 phosphorylation levels did not significantly differ between the Scr control, Sh-APP and Shres-APP_{WT} conditions which is to be expected. Indeed, since under healthy conditions the majority of APP is cleaved by α- followed by γ-secretase in the non-amyloidogenic pathway (O'Brien and Wong, 2011), knocking down APP or expressing it at endogenous levels would not significantly affect these negligible levels of Aβ since there is no overexpression of APP or increase in APP amyloidogenic processing.

Cortactin is the target of SFK phosphorylation on residues Y421, Y466 and Y482 (Huang *et al*., 1997a; Huang *et al*., 1998), and this acts to regulate a number of cellular events (Huang *et al*., 1997a; Huang *et al*., 1997b; Martinez-Quiles *et al*., 2004; Cao *et al*., 2009; Vivstein and Pthenveedu, 2014). Importantly, the phosphorylation of cortactin C-terminal tyrosine residues negatively regulates the lysosomal targeting of GluA2-containing AMPARs (Parkinson *et al*., 2018). Indeed, mutating all three tyrosines on cortactin to be phospho-null increases GluA2 binding, therefore phosphorylation at one or all of these tyrosines reduces GluA2 binding so that cortactin can no longer maintain surface levels of GluA2/3 heteromers and receptors are targeted to lysosomes. Since our data suggest that phosphorylation of cortactin at Y466 was increased in response to amyloidopathy, it is possible that this mechanism contributes to GluA2/3 lysosomal targeting in AD.

6.4.5 Trafficking of GluA2/3 heteromers

There are predominantly two distinct populations of AMPARs in the adult hippocampus, GluA1/2 and GluA2/3 (Wenthold *et al*., 1996). Since we demonstrated that there was no significant reduction in GluA1 expression levels (figure 5-2 B, figure 5-4 D) and no significant co-localisation of GluA1 with LAMP1 (figure 6-3 B, figure 6-4 B), the results suggest that trafficking of GluA1-containing receptors are not disrupted by amyloidopathy. Thus, the pool of GluA2 affected in the disease state is likely to be in complex with GluA3. Indeed, in a previous study, knocking down cortactin was shown to significantly reduce expression of GluA3 as well as GluA2, and increase the co-localisation of GluA3 and GluA2 with LAMP1, suggesting that cortactin is essential for maintaining surface levels of GluA2/3 heteromers (Parkinson *et al*., 2018). This supports a model in which GluA2/3 heteromers

traffic to lysosomes when cortactin is phosphorylated in response to amyloidopathy. As mentioned previously, the GluA3 subunit has been previously shown to promote lysosomal targeting (Lee, Simonetta and Sheng, 2004) and there is evidence that mice lacking GluA3-containing AMPARs are protected against Aβ-induced synaptic deficits (Reinders *et al*., 2016). Thus, it may be the case that the GluA3 subunit is predominantly responsible for GluA2/3 lysosomal sorting. Previous work in our lab has demonstrated that cortactin can bind the GluA3 C-terminal tail. It would be interesting to investigate this interaction further to determine whether or not a cortactin-GluA3 interaction can also regulate GluA2/3 lysosomal targeting, and whether this is disrupted in the disease state.

6.4.6 Fyn kinase in AD

A variety of kinases in the SFK family have been implicated in the phosphorylation of cortactin, such as Fer, Fyn, Nck1, Src and Syk (Wu and Parsons, 1993; Kim and Wong, 1998; Gallet *et al*., 1999; Kapus *et al*., 2000; Fan *et al*., 2004; Oser *et al*., 2010). However, Fyn kinase is of particular interest in this model since it has previously been implicated in the phosphorylation of cortactin (Kapus *et al*., 2000; Huang *et al*., 2003; Janjanam and Rao, 2016) and it has demonstrated aberrant activity in response to Aβ. Indeed, the first evidence for a direct involvement of Fyn in AD came in 1998 when slices from Fyn knockout mice were spared of Aβ-mediated toxicity (Lambert *et al*., 1998), and since then APP transgenic mouse models have demonstrated that Fyn overexpression promotes Aβ-induced synaptotoxicity, a phenomenon which can be rescued by Fyn ablation (Chin *et al*., 2004, Chin *et al*., 2005). Furthermore, exposing cultured neurons to Aβ resulted in increased levels of phosphorylated Fyn, and Fyn kinase was required for Aβ-induced spine loss (Um *et al*., 2012). The robust evidence for the aberrant activation of Fyn in response to amyloidopathy suggests that this specific member of the SFK family could be responsible for the increased cortactin Y466 phosphorylation in the disease state.

This chapter demonstrated that GluA2-containing AMPARs are targeted for lysosomal degradation following APP_{WT} overexpression. However, the overall size of this effect was small, possibly due to lysosomal dysfunction that can occur following Aβ pathology (Knauer *et al*., 1992; Yang *et al*., 1998; Ditaranto, Tekirian and Yang, 2001; Liu *et al*., 2010b) or because of the involvement of additional degradative systems such as the UPS (Claque and Urbé, 2010). Nevertheless, cortactin phosphorylation was increased in response to APP_{SWE} expression, specifically at residue Y466. Since cortactin Y466 phosphorylation disrupts the cortactin-GluA2 interaction and the maintenance of GluA2/3 surface levels (Parkinson *et al*., 2018), the results suggest that this mechanism could be

operating in response to amyloidopathy to increase GluA2/3 lysosomal targeting. The aberrant activation of Fyn kinase has been previously demonstrated in response to Aβ (Lambert *et al*., 1998; Chin *et al*., 2004, Chin *et al*., 2005; Um *et al*., 2012) and fyn kinase has previously been implicated in the phosphorylation of cortactin (Kapus *et al*., 2000; Huang *et al*., 2003; Janjanam and Rao, 2016), making it likely that this kinase is responsible for increased cortactin phosphorylation in the disease state.

7 General Discussion

7.1 Summary

The data presented in this thesis demonstrate that there is a reduction in the expression of the AMPAR subunit GluA2 in response to amyloidopathy. The results suggest Aβ reduces the efficiency of GluA2-containing AMPAR recycling, so that these receptors are sorted into lysosomes where the cargo is degraded. The data also demonstrate that there is an increase in cortactin phosphorylation at residue Y466 in response to amyloidopathy, which suggests that Aβ could be causing AMPAR lysosomal targeting through the disruption of the cortactin-GluA2 interaction, which functions to maintain surface levels of GluA2/3 heteromers (Parkinson *et al*., 2018) (figure 7-1). This work supports previous observations that AMPARs are lost from synapses in AD models (Hsieh *et al*., 2006; Liu *et al*., 2010; Zhao *et al*., 2010; Miñano-Molina *et al*., 2011; Alfonso *et al*., 2014) and that, generally speaking, there is a reduction in AMPAR protein levels in post-mortem AD brains compared to controls (Armstrong *et al*., 1994; Yasuda *et al*., 1995; Ikonomovic *et al*., 1997; Carter *et al*., 2004), and describes the first identified mechanism for the loss of AMPAR subunit expression in the disease state. The wider implications and future directions that these results present will be discussed below.

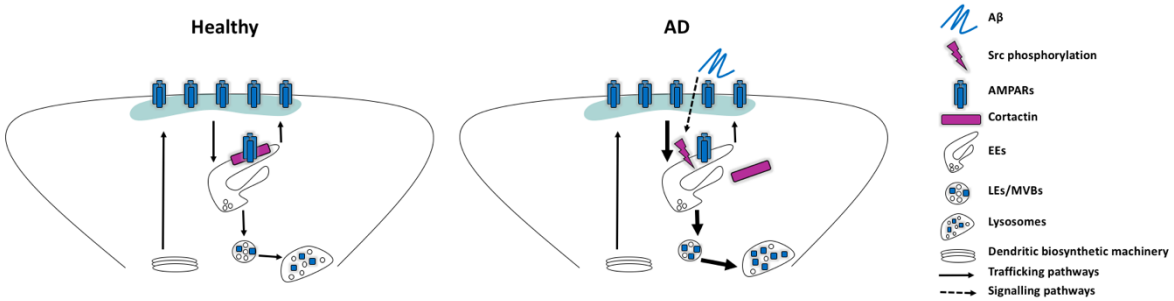

Figure 7-1 Schematic of the proposed role of Aβ in disrupting AMPAR trafficking.

Under healthy conditions, a strictly regulated balance of constitutive AMPAR internalisation and recycling maintains a constant number of AMPARs at the post-synaptic membrane. Most endocytic events are likely to result in their return to the plasma membrane. However, a number of these events will result in AMPAR degradation, with EEs maturing into late endosomes and lysosomes. The degraded receptors are presumably replaced by newly synthesised AMPARs from the biosynthetic machinery. The results presented in this thesis indicate that Aβ functions to disrupt the efficient recycling of AMPARs back to the synapse. Indeed, there is a restriction in AMPAR recycling which is thought to be mediated by GluA2-containing receptors. Aβ can activate SFKs, which phosphorylate cortactin and cause the dissociation between cortactin and

GluA2-containing AMPARs. This results in less efficient GluA2-containing AMPAR recycling, resulting in more lysosomal AMPAR degradation.

7.2 WIDER IMPLICATIONS AND FUTURE DIRECTIONS

7.2.1 Modelling Alzheimer's disease

Experimental models of AD are vital to better understand the pathophysiology of the disorder and for testing novel therapeutics. However, success in AD clinical trials has been limited thus far due to poor translation of successful preclinical testing in animal models into efficacious treatments (Banik *et al*., 2015). A plethora of models exist encompassing both transgenic mice and *in vitro* brain tissue. Since each model replicates different pathological hallmarks of the disease, gaining consistent results has proved troublesome. In this thesis, AMPAR subunit expression was examined in 3 models of AD: the overexpression of APP_{WT} in neuronal culture, the expression of APP_{SWE} in neuronal culture and the J20 transgenic mouse model.

In the past, researchers have struggled to generate models of AD that overproduce Aβ without overexpressing APP, therefore in order to mimic Aβ pathology many research groups have utilised models that overexpress human WT APP (Kamenetz *et al*., 2003; Hsieh *et al*., 2006; Simón *et al*., 2009) or APP with human mutations (Gu, Liu and Yan, 2008; Cantanelli *et al*., 2014). However, increasing gene expression beyond physiological levels can introduce artefacts (Kuang *et al*., 2006). Thus, some researchers believe that APP overexpression is an artificial means to model AD. In order to overcome these issues, our lab developed a model of AD that replaces endogenous APP with a clinically relevant familial mutation, APP_{SWE}, in culture. During this project, we then developed a control by cloning a virus that replaces the endogenous protein with APP_{WT}. This allowed us to use these viruses for the first time to explore molecular mechanisms of the disease. It will be vital to confirm that the APP_{SWE} is overproducing $A\beta_{42}$ in future studies by using an Aβ-production assay of culture media with anti-Aβ antibodies, but this novel approach to model amyloidopathy shows promise as a cell culture model of the disease.

Transgenic mouse models dominate approaches to the animal modelling of AD, since they allow many critical aspects of the disease to be reproduced through the expression of familial genetic mutations. However, as a complex disorder, AD is a polygenic and multifactorial disease in which

hundreds of defective genes may contribute to its pathogenesis (Cacabelos *et al*., 2014). As such, the exact pathology and phenotype of each transgenic strain depends on the familial AD (fAD) mutation, the promoter used and the background mouse strain (Drummond and Wisniewski, 2017). Indeed, the degree to which each model is characterised in terms of the presence of plaques, gliosis, neurofibrillary tangles, neurodegeneration, synaptic failure and the extent of cognitive impairment varies greatly (Higgins and Jacobsen, 2003). Thus, although J20 models in this instance did not display AMPAR expression loss, this phenotype has been demonstrated in other transgenic strains (Baglietto- Almeida *et al*., 2005; Zhang *et al*., 2011; Cantanelli *et al*., 2014; Vargas *et al*., 2018). It might be that the specific genetic make-up of the J20 strain is less vulnerable to AMPAR pathology, although the results could have been due to negligible levels of APP overexpression in some of the transgenic mouse brains.

Since the vast majority of AD transgenic models have pathology that is dependent on the expression of fAD mutations and most AD clinical trials are conducted in sporadic AD (sAD) patients with pathogenesis that has distinctions from fAD, this represents one stumbling block for the translatability of success (Drummond and Wisniewski, 2017). Indeed, developing a practical and widely available model of sAD would be indispensable for understanding molecular mechanisms and advancing pre-clinical studies, and it would be useful to examine whether AMPAR pathology exists here.

One way to do this could be through the use of induced pluripotent stem cells (iPSCs). Indeed, since their discovery in 2006 by Yamanaka and colleagues, much progress has been made to develop clinically relevant cell culture systems (Takahashi and Yamanaka, 2006). The technique allows a patient's somatic cells to be reprogrammed to a pluripotent state by the forced expression of a set of transcription factors (Majolo *et al*., 2019) and was implemented in AD for the first time in 2011 with the successful differentiation of these cells into neurons (Yagi *et al*., 2011; Yahata *et al*., 2011). iPSCs offer great potential to model AD *in vitro* since they can be generated from both sAD and fAD patients and could allow treatment to be individualised based on the behaviour of the cellular model (Majolo *et al*., 2019). Furthermore, genome editing such as clustered regularly interspaced short palindromic repeats (CRISPR) can be used to correct mutations in cells from fAD patients or to introduce mutations into cells from healthy control subjects (de Leeuw and Tackenberg, 2019). Even sAD cases can be modelled by introducing AD risk genes such as *APOE*. Indeed, iPSCs provide a platform to better understand disease mechanisms in human cells and discover novel therapeutics (de Leeuw and Tackenberg, 2019).

7.2.2 Reduced GluA2 expression in response to amyloidopathy

Along with an overall reduction in synaptic AMPAR number, one of the possible outcomes of the specific loss of GluA2/3 heteromers is the reduction in GluA2 content at the synaptic plasma membrane and the consequent expression of CP-AMPARs. The mechanisms by which CP-AMPARs contribute to neurodegeneration in AD have not been elucidated, but since under physiological conditions CP-AMPARs appear only transiently in response to synaptic activity (Plant *et al*., 2006) it is likely that aberrant activation of these receptors in the disease state leads to excitotoxicity, a pivotal catalyst for the onset of pathology.

For future directions of this thesis, it would be useful to examine CP-AMPAR expression and insertion in our cell culture models. This would provide further support for a model in which GluA2-containing AMPARs are targeted for lysosomal degradation in response to Aβ, leaving GluA2-lacking CP-AMPARs to be inserted at the surface. The incorporation of CP-AMPARs at the surface membrane is accompanied by an increase in inward rectification, a well established assay for the absence of GluA2-containing AMPARs (Hume, Dingledine and Heinemann, 1991). Recording AMPAR-mediated EPSCs in hippocampal brain slices expressing amyloidogenic APP would determine the extent of inward rectification in their current-voltage relationships. The presence of this distinct electrophysiological phenotype would provide further support for the expression of GluA2-lacking CP-AMPARs at the cell surface.

7.2.3 Mechanisms of GluA2 expression loss

The recycling of receptors to the plasma membrane is recognised as a complex process that consists of both sequence-dependent, actively-regulated and passive sequestration of cargo into tubular domains on early endosomes (EEs) (Puthenveedu *et al*., 2010). There is significant overlap between endolysosomal and ubiquitin-mediated degradation, and the dysregulation of these systems has been consistently observed in neurodegenerative diseases (Nedelsky, Todd and Taylor, 2008; Lee, Lee and Rubinsztein, 2013). Indeed, other than AD, endosomal dysfunction is an early indicator for a number of neurodegenerative diseases such as Parkinson's disease (Schreij, Fon and Mcpherson, 2015), Niemann-Pick type C1 (D'Arcangelo *et al*., 2011; Rabenstein *et al*., 2017) and neuropathologies such as ischemia (Yuan, Liu and Hu, 2017). This thesis proposes a novel mechanism of endolyosomal dysfunction in AD, whereby Aβ reduces the efficiency of GluA2-containing AMPAR

recycling, so that these receptors are sorted into LEs and lysosomes where the cargo is degraded. As a result, fewer receptors are recycled back to the post-synaptic membrane via REs. In order to extend our investigations in the future, this hypothesis could be further tested by examining AMPAR localisation in other endosomal compartments for example those positive for the recycling endosomal marker, Rab11. Indeed, if amyloidopathy did regulate GluA2-containing AMPAR endolysosomal targeting, these experiments would be expected to show a decreased co-localisation between internalised GluA2 and Rab11.

Although it was demonstrated that cortactin phosphorylation at Y466 was increased in response to APP_{SWE}, no causal relationship between Y466 phosphorylation and GluA2 lysosomal targeting was demonstrated. Future directions of this work could utilise the overexpression of a phosphonull mutant, Y466A-cortactin, to validate this. If cortactin phosphorylation is indeed responsible for GluA2 lysosomal degradation, this mutant should prevent the co-localisation of GluA2 with LAMP1 and consequent degradation.

It would also be of interest to determine the kinase responsible for aberrant cortactin phosphorylation during Aβ pathogenesis. A variety of kinases in the SFK family have been implicated in the phosphorylation of cortactin, such as Fer, Fyn, Nck1, Src and Syk (Wu and Parsons, 1993; Kim and Wong, 1998; Gallet *et al*., 1999; Kapus *et al*., 2000; Fan *et al*., 2004; Oser *et al*., 2010). However, Fyn kinase is of particular interest in this model since it has demonstrated increased activity in response to Aβ (Lambert *et al*., 1998; Chin *et al*., 2004, Chin *et al*., 2005; Um *et al*., 2012). The robust evidence for the aberrant activation of Fyn in response to amyloidopathy suggests that this specific member of the SFK family could be responsible for the increased cortactin Y466 phosphorylation in the disease state. In order to investigate this, aberrant Fyn activity could be examined through Western blotting for levels of Y416-Fyn in response to APP_{SWE} expression, since Fyn is phosphorylated at this tyrosine residue following activation (Um *et al*., 2012). Increased levels of Y416-Fyn would indicate that Fyn kinase might be responsible for increased cortactin phosphorylation in this model. Alternatively, expressing a kinase-dead mutant of Fyn would determine if the increase in cortactin Y466 is blocked.

7.2.4 Limitations

One limitation of our antibody feeding and immunocytochemistry experiments was that appropriate controls were not implemented. These should be carried out in future experiments and include primary antibody controls to confirm the specificity of the primary antibody binding to the antigen. This can be carried out by pre-incubating the antibody with blocking peptides, which should

inactivate the antibody so that the cultures show little or no staining (Burry, 2011). A secondary antibody control should also be carried out to show that the labelling observed is due only to binding of the secondary antibody to the primary antibody. This control can be carried out by eliminating the primary antibody to check for any nonspecific binding, since no labelling should be seen in this condition (Burry, 2011). A labelling control is an additional control which should be carried out by examining cultures under the microscope under the same conditions but with no antibodies to demonstrate the labelling is not the result of endogenous labelling or reacting products (Burry, 2011).

Additionally, it would be advantageous to confirm that the AMPAR complexes bound to antibody in our experiments follow the same path from the EE as AMPAR complexes not bound to antibody. This could be done via electrophysiology with the use of drugs to block lysosomal targeting, or via biochemical techniques that involve fractioning endosomal compartments by differential ultracentrifugation.

Another limitation was that, due to time constraints, western blotting experiments had to be carried out on cortical cultures. Given a model for the specific loss of GluA2/3 heteromers has previously been proposed in response to ischaemia in hippocampal but not cortical neurons involving a cell-type specific mechanism, (Koszegi *et al*., 2017) it would have been ideal to carry out all experiments in hippocampal neuronal cultures. However, our results demonstrated that a loss of GluA2 expression was demonstrated in both cortical and hippocampal *in vitro* models of AD, indicating the involvement of molecular cell biology that is common to both cell types.

7.3 CLINICAL APPLICATIONS

There have been major efforts in recent years to establish the cellular correlate of learning and memory, with much evidence now suggesting that synaptic plasticity, specifically the LTP of excitatory synaptic transmission in the hippocampus, is critical for the formation of long-term memories (Morris *et al*., 1982; Morris *et al.,* 1986; Tsien Huerta and Tonegawa, 1996; Nakazawa *et* al., 2002; Whitlock *et* al., 2006; Hunsaker, Lee and Kesner, 2008). The major expression mechanisms of NMDAR-dependent LTP involve changes to the biophysical properties of AMPARs, as well as their trafficking to the cell surface (Derkach, Barria and Soderling, 1999; Hayashi *et al*., 2000; Lee *et al*; 2000; Nicoll, 2017; Diering and Huganir, 2018). It is no surprise then that AD, now considered to be primarily a disorder of synaptic failure, is characterised by cognitive impairment which typically

begins with memory deficits and eventually develops to incapacitating levels (Alzheimer, 1907). Indeed, a host of literature has demonstrated a role for Aβ in promoting the loss of surface AMPARs (Hsieh *et al*., 2006; Liu *et al*., 2010; Zhao *et al*., 2010; Miñano-Molina *et al*., 2011; Alfonso *et al*., 2014), which manifests as synaptic depression and deficits in learning and memory (Anggono and Huganir, 2012). Preventing the loss of these receptors is thus vital in combatting cognitive detriment.

Indeed, reducing processes that regulate AMPAR ubiquitination and degradation have already been shown to rescue Aβ-induced synaptic deficits (Rodrigues *et al*., 2016). If cortactin does in fact regulate GluA2-containing AMPAR degradation in response to amyloidopathy, it may prove to be a novel therapeutic target to combat AMPAR loss and cognitive decline. Furthermore, considering the selective loss of the GluA2 subunit from the cell surface has been witnessed in other disease states including Parkinson's disease (Diogenes *et al*., 2012; Cortese *et al*., 2016) and ischaemic brain injury (Dixon, Mellor and Hanley, 2009; Blanco-Suarez and Hanley, 2014), investigating the role of lysosomal degradation and the cortactin-AMPAR interaction in these cases could define a ubiquitous mechanism responsible for AMPAR loss across multiple neurodegenerative disorders.

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